Dose Dependence of Immunopotentiation and Tumor Regression Induced by Levamisole

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SUMMARY

Breast cancer was induced in female Sprague-Dawley rats by 7,12-dimethylbenz(a)anthracene. Once tumors had become established, they were treated with varying doses of the immunopotentiating drug, levamisole. Tumor growth was measured in the various dosage groups, and at 6 months after tumor induction the animals were sacrificed. Their immunological competence at this time was measured by the mitogen responses of splenic lymphocytes.

Untreated animals with breast cancer were found to be immunosuppressed compared to normal animals. The drug levamisole resulted in immunopotentiation, but at high doses it was immunosuppressive. Tumor regression was observed at doses that resulted in immunopotentiation, but not at high doses. There was a significant correlation between immune competence and tumor regression. It is concluded that levamisole can cause regression of breast cancer in the rat but that this effect is critically dependent on the dose of the drug; these observations confirm previous studies carried out on human cells in vitro. It is recommended that high doses of the drug be avoided in human clinical trials and that the patients who receive this drug should have their immune responses carefully monitored.

INTRODUCTION

The anthelmintic drug, levamisole, has been shown to potentiate cellular immunity (5, 7, 8, 10, 14); since it is a relatively nontoxic thiazole derivative (Chart 1), this property has led to clinical trials in human breast cancer (11) and in other cancers (1, 14, 15). However, in animal tumor systems the efficacy of the drug has been variable. Thus, the dose of the drug; these observations confirm previous studies carried out on human cells in vitro. It is recommended that high doses of the drug be avoided in human clinical trials and that the patients who receive this drug should have their immune responses carefully monitored.

In a recent study (13) we found that the immunopotentiating effect of levamisole on human lymphocytes in vitro was critically dose dependent and that, while at optimum dose levels both mitogen responses and reactivity in mixed lymphocyte culture could be increased, at high doses the drug was immunosuppressive. This led to the realization that, in clinical trials, potential harm could be done to patients with cancer if immunosuppressive doses were given. These observations led to the present study in which we sought to confirm similar dose-response relationships in the potentiation of immune responses in vivo in an animal model and in which we wished to determine whether the antitumor activity of the drug correlated with its effect on immune responses.

The study began with the induction of breast cancer in female Sprague-Dawley rats by means of DMBA. Following the appearance of tumor, the animals were divided into groups that received varying doses of levamisole p.o. At the end of a 6-month period the tumors were measured and the animals were sacrificed. The immune competence of the animals in the various dosage groups was monitored by assaying mitogen responses of splenic lymphocytes.

MATERIALS AND METHODS

One hundred fifty Sprague-Dawley rats were obtained; 30 of these received no treatment and served as normal control animals. The remaining 120 animals were given 20 mg DMBA on the 56th day of life and then divided into 4 groups of 30 animals each. One group was given no therapy following the induction of breast cancer with DMBA, and the remaining 3 groups were given levamisole p.o. in doses of 2, 4, and 8 mg/kg body weight daily once they had developed tumors measuring at least 0.5 cm in diameter. Each animal was evaluated twice weekly for weight gain and presence and size of tumor. Tumor regression was defined as a decrease of at least 0.5 cm in diameter. At the end of 6 months the final sizes of the tumors were measured, and the animals were sacrificed. The mitogen studies were then carried out on a sample of all the groups including the normal controls, the rats with untreated cancer, and those in the various treatment dosage regimens.

The mitogen studies were carried out using splenic lymphocytes. After anesthesia with ether, the spleen was removed and approximately one-half was diced into small cubes that were then homogenized in a TenBroeck homogenizer using supplemented Eagle's minimal essential medium as a suspending fluid. The Eagle's minimal essential...
medium was supplemented with 10% heat-inactivated human AB serum, 100 units penicillin per ml, 100 μg streptomycin per ml, 1% L-glutamine (200 mM; Microbiological Associates, Bethesda, Md.), and 2.5% N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid buffer (1 mM; Grand Island Biological Co., Grand Island, N. Y.).

We adjusted cell concentration to 5 x 10⁶/ml with Eosin-Y to determine viability and gentian violet for the final counting. Two-tenths ml of the cell suspension was placed in the wells of Linbro microtiter plates (Linbro Chemical Co., New Haven, Conn.). The response to 3 mitogens was assayed using 10 μl of a 1:1 dilution of a 5-ml stock ampul of PHA (Difco Laboratories, Detroit, Mich.), 10 μl of a 1:150 dilution of Con A stock solution of 10 mg/ml (Difco), or 10 μl of an undiluted solution of 1 ampul of PWM in 5 ml (Grand Island Biological Co.). As controls, cells were also incubated in the absence of mitogen. All studies were run in triplicate. After 3 days of incubation at 37° in a humidified atmosphere containing 5% CO₂, 2 μCi [³H]thymidine were added to each well, and the plates were incubated for a further 5 hr. At the end of this period, the cells were harvested with the multiple automated sample harvester (Mash II Model, Microbiological Associates) onto Whatman glass fiber discs (H. Reeve Angel & Co., Clifton, N. J.). The discs were then dried, immersed in scintillation fluid, and counted on a Nuclear-Chicago counter. Mean counts were calculated for the triplicate runs.

The mitotic index for each of the 3 mitogens was calculated from the formula

\[
\text{Mitotic index} = \frac{\text{cpm for cells + mitogen}}{\text{cpm for cells alone}}
\]

The mean values for tumor size and mitotic index for each group of animals were compared by the Student t test, and tumor size was correlated with mitotic index by linear regression analysis.

RESULTS

The rate of growth of the tumors is illustrated in Chart 2. Those rats that did not receive levamisole developed tumors that grew rapidly during the 15-week observation period. The tumors first appeared between 6 and 8 weeks after the rats were given DMBA. Those animals that were given levamisole, 2 or 4 mg/kg, showed a clear pattern of inhibition of tumor growth after 6 weeks of tumor age. After this time the tumors were consistently smaller than those seen in the controls (p < 0.005). However, those animals that received levamisole, 8 mg/kg, showed no inhibition of tumor growth and, in fact, tended to have larger tumors than did the controls, although this was not statistically significant. The decrease in size of the tumors in animals on the high dose of levamisole that was observed toward the end of the study was simply a reflection of the fact that many of these animals died of their cancer at this time, these being the ones with the largest tumors.

Table 1 is a summary of the state of the animals at the end of a 6-month period from tumor induction. Animals that did not receive DMBA did not develop tumors, whereas two-thirds of the animals given this carcinogen developed breast cancer. There was no evidence of tumor regression in untreated animals whereas, in contrast, many animals given levamisole, 2 or 4 mg/kg, showed evidence of tumor regression as measured by disappearance or decrease in size. However, there was no instance of tumor regression in those animals treated with high doses of levamisole at 8 mg/kg. Deaths in the control group and among those animals that did not develop tumors were due to pneumonia.

The relationship between mean tumor diameter at the end of the study and dose of levamisole is shown in Chart 3, and it shows that the optimum dose of levamisole is 2 to 4 mg/kg. Doses in excess of this do not inhibit tumor growth.

The effect of different doses of levamisole on cellular immunity as measured by responses of splenic lymphocytes to vegetable mitogens is shown in Table 2. The values given are cpm/culture and are the mean of triplicate observations in each experiment. The mitotic indices calculated from the data shown in Table 2 for the 3 mitogens PHA, Con A, and PWM were plotted against the dose of levamisole that the animals received (Chart 4). Chart 4 shows that untreated animals with breast cancer have slightly impaired responses compared with normal animals, whereas those animals that received doses of levamisole between 2 and 4 mg/kg show evidence of immunopotentiation. This was most evident when Con A was used as the mitogen, suggesting activity on a T-cell population.
Effect of levamisole on state of animals with breast cancer at end of 6-month observation period

<table>
<thead>
<tr>
<th>Animal</th>
<th>Total no.</th>
<th>No. alive</th>
<th>No. with tumor</th>
<th>No. with regression</th>
<th>No. dead with tumor</th>
<th>No. dead without tumor</th>
<th>Size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal animal</td>
<td>30</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Untreated breast cancer</td>
<td>30</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>11</td>
<td>9</td>
<td>8.5</td>
</tr>
<tr>
<td>Levamisole, 2 mg/kg</td>
<td>30</td>
<td>21</td>
<td>12</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>1.9</td>
</tr>
<tr>
<td>Levamisole, 4 mg/kg</td>
<td>30</td>
<td>23</td>
<td>9</td>
<td>10</td>
<td>2</td>
<td>5</td>
<td>1.7</td>
</tr>
<tr>
<td>Levamisole, 8 mg/kg</td>
<td>30</td>
<td>23</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>6.9</td>
<td></td>
</tr>
</tbody>
</table>

* Mean diameter of all tumors in the respective groups.

The relationship between mitotic index for the 3 mitogens and tumor diameter is shown in Chart 5. There was an excellent linear negative correlation between immune competence and tumor size for all mitogens (for Con A, \( r = -0.98, p < 0.001 \); for PHA, \( r = -0.88, p < 0.05 \); and for PWM, \( r = -0.94, p < 0.01 \)).

**DISCUSSION**

This study was stimulated by the realization that the immunopotentiating effect of levamisole was critically dose dependent and that high doses of the drug could be immunosuppressive. This was the result of a previous investigation (13) in which we showed that levamisole could potentiate a mixed-lymphocyte culture or the response of human peripheral blood lymphocytes to mitogens in vitro but that, in this system, immunosuppression occurred at high doses. The present study confirms the importance of the dose of levamisole. In a dose range of 2 to 4 mg/kg, there was clear evidence of inhibition of tumor growth and even evidence of...
tumor regression; but at higher doses this was not observed and, in fact, the spread of cancer tended to be worse than that in untreated animals. This correlated very well with the immune competence of the animals in a negative way; i.e., those animals showing the greatest immunopotentiation had the least tumor mass. In this study, tumor mass was measured in terms of mean tumor diameter, but when this is converted to volume the effects are even more marked since tumor mass variation would be raised 3 logs

These observations do not prove that tumor inhibition was a direct result of immunopotentiation induced by levamisole, but this is the most likely explanation of the results. It could be argued that the drug itself has antitumor activity, but this is unlikely for 2 reasons: (a) direct antitumor activity of levamisole has not been reported; and (b) the immunopotentiation seen to accompany decrease in tumor size is unlikely to be due to direct removal of tumor since the immune competence of the optimum treatment groups was well above normal.

Our observations explain, in part, some of the controversy surrounding the use of levamisole in animal tumor systems. Several of the reports indicating a lack of effect of the drug (4, 6) were based on studies in which high doses of the drug were used, and it is likely that these doses were immunosuppressive. On the other hand, those studies that demonstrate antitumor activity used lower doses of levamisole (3, 12).

In addition, varying routes of administration of the drug have been used, and there are almost certainly variations in the antigenicity of the tumor systems studied.

The critical nature of the dose of levamisole may represent a more general biological phenomenon that has considerable importance in attempts to manipulate immune responses. It has been observed that many dose-response relationships that modify the activity of the reticuloendothelial system are not linear but frequently result in the production of biphasic M- or W-shaped curves (2). Our observations may be a specific instance of such a phenomenon that reflects activity on separate cell populations or interference with negative feedback control systems.

The optimum dose of levamisole indicated by this study appears to be 2 to 4 mg/kg, and this agrees closely with the recommended human dose of approximately 150 mg/day. The dose-response curves for mitotic activity in the rat bear a striking resemblance to those that we previously reported (13) in the in vitro human studies.

In view of the increasing development of clinical trials of levamisole in human cancer and the resultant encouraging results thus far obtained (11), we believe that it is of great importance that recommended dose levels of the drug not be exceeded for fear of inducing an immunosuppressive state. Ideally, all such clinical trials should include some monitor of immune competence in the patient in order to demonstrate that the objective of immunopotentiation is, in fact, being achieved.

**REFERENCES**

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