The immunological evaluation of levamisole treatment in cancer patients

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Summary
The effect of levamisole on different immunological parameters was studied in patients with various malignant diseases.

Levamisole restored tuberculin-negative delayed hypersensitivity reactions and increased the sensitivity to 1-nitro-2,4-dichlorobenzene challenges. It also increased low E-rosette formation of T-lymphocytes and enhanced the clearance of a lipid emulsion by the reticulo-endothelial system.

Levamisole had no effect on serum immunoglobulin levels but increased serum haemolytic complement activity.

Introduction
In recent years the occurrence of humoral and cellular immune reactions against tumour antigens has been well established. The immune status seems to be normal in patients with early cancer, whilst it may be depressed in patients with advanced cancer as measured by delayed hypersensitivity reactions (Anthony et al., 1974; Eilber and Morton, 1970), lymphoblast transformation tests (Cheema and Hersch, 1971; Whittaker, Rees and Clark, 1971; Suci-Foca et al., 1973; Twomey, Catalona and Chretien, 1974) or by the E-rosette test (Anthony et al., 1975; Dellon, Potvin and Chretien, 1975; Elhilali et al., 1976; Gross et al., 1975; Whitehead et al., 1976; Wybran and Fudenberg, 1973). Since depressed immunity seems to be associated with a bad prognosis, immunotherapy might well be a rational approach in cancer therapy. Different modes of immunotherapy—passive, adoptive, active—have been proposed, excellently reviewed by Bluming (1975). Of these, the non-specific active therapy with B.C.G. seems to be a value although results are controversial and side effects are a serious disadvantage. Other non-specific active immunotherapeutics currently under evaluation are Corynebacterium parvum, polyinosinic-cytidylic acid and, very recently, levamisole.

Renoux and Renoux (1971) reported that the anthelmintic drug levamisole increased the protective effect of a bacterial vaccine in mice. Two years later the present authors found that a 3-day treatment course with levamisole could restore delayed hypersensitivity reactivity to purified protein derivative (PPD) in patients with various debilitating diseases (Verhaegen et al., 1973; Brugmans et al., 1973). Since then, the effects of levamisole on different parameters of humoral and cellular immunity have been extensively studied and excellently reviewed by Oettgen, Pinsky and Delmonte (1976) and Symoens and Rosenthal (1977).

In tumour-bearing animals (Chirigos, Pearson and Fuhrman, 1974; Chirigos, Fuhrman and Pryor, 1975; Perk et al., 1975) and cancer patients (Amery, 1976; Rojas et al., 1976) levamisole therapy has shown promising results on the course of the disease. Experience with levamisole in human tumour immunology is now reported.

Materials and methods
Delayed hypersensitivity reaction to tuberculin
Sixty tuberculin-negative patients with various malignant diseases were selected at random. Forty were treated with a single oral dose of 150 mg of levamisole for 3 consecutive days and twenty with placebo.

Tuberculin response was measured by the
Mantoux test. Ten i.u. of PPD* were injected intradermally and readings were taken 48 hr later without knowledge of the medication. A test was considered positive when the diameter of induration was at least 5 mm.

The interval between the selection and the subsequent test was one week. The first dose of levamisole was given 48 hr after the first PPD injection.

Delayed hypersensitivity reaction to dinitrochlorobenzene

One hundred and five patients with various malignant diseases were sensitized with 2 mg of 1-nitro-2,4-dichlorobenzene in aerosol (DNCB) and challenged with 200, 100, 50, and 25 μg DNCB in aerosol 14 days later. Readings were taken after 48 hr and a test was considered positive for a given dose when the diameter of induration was at least half as large (≥7.5 mm) as the sensitized area.

Thirty-nine patients received 150 mg of levamisole/day for the time of follow-up, forty-one patients received 150 mg of levamisole for 3 consecutive days repeated every fortnight and twenty-five patients received placebo daily.

When the patients were seen again in the outpatient department, they were challenged with those doses of DNCB to which they were previously negative. The observer did not know the medication.

Forty-seven patients treated with levamisole could be followed-up for more than one year.

Lipofundin clearance test

The lipofundin clearance test was performed on twelve cancer patients before and 1–2 weeks after treatment with 150 mg of levamisole/day. Measurements were performed blind.

This test was performed using a modified method after Lemperlee, Reichelt and Denk (1971). Fifty ml of lipofundin-S-20 (B. Braun, Melsungen, Germany) was injected intravenously within 2 min and blood samples of 1-6 ml in 0.4 ml citrate were taken before and after 2, 4, 6, 8 and 10 min of injection. After dilution with 4 ml saline, the samples were centrifuged for 2 min at 2500 rev/min and after appropriate dilution the turbidity of the supernatant was measured spectrophotometrically.

The half-life of the lipid (T_{1/2}) was calculated on a semi-logarithmic chart.

Immunoglobulins

In eighty cancer patients, serum levels of IgA, IgG and IgM were determined before and after treatment with 150 mg of levamisole daily for various periods.

* Albumose-free tuberculin, Swiss Serothapeutic and Vaccine Institute, Bern (Switzerland).

of time ranging from 1 week to 4 months. Measurements were performed blind. The radial immunodiffusion technique of Mancini, Carbonara and Heremans (1965) was used with commercial plates and standards (Behringwerke AG).

Serum complement

The study was composed of ninety-one cancer patients; fifty-eight were treated with a single oral dose of 150 mg of levamisole daily for 1–2 weeks and thirty-three were treated with placebo.

Haemolytic complement activity (CH50) was determined by a modified method of Mayer (1971) and complement components C3, C4 and C1q by the radial immunodiffusion technique of Mancini et al. (1965) as described by Verhaegen et al. (1976).

E-rosette forming cells

Sixty-two patients with various malignant diseases participated in the study. Thirty-four were treated with a single oral dose of 150 mg levamisole daily for one week and twenty-eight patients were similarly treated with a placebo. Peripheral E-rosette forming cells (RFC) were assessed before and after treatment as described by Verhaegen et al. (1977).

Allocation of the patients

The patients were randomly allocated to placebo and levamisole groups, but randomization was not fully stratified by tumour type. In the delayed hypersensitivity studies and the complement study a representative number of patients, i.e. about one-third of the total number of selected patients, were allocated to the placebo groups. In the E-rosette study about equal numbers of patients were allocated to placebo and levamisole groups.

Concomitant treatment

In all studies reported, patients were not allowed to take cytostatic drugs, except the forty-seven patients in the DNCB study who were followed-up for more than one year.

Statistical analysis

Intergroup differences were analysed by the Mann–Whitney U test (Siegel, 1956a). Intragroup differences were analysed by the Wilcoxon matched-pairs signed-ranks test (Siegel, 1956b).

Results

Delayed hypersensitivity reaction to tuberculin

Out of forty tuberculin-negative cancer patients treated with 150 mg of levamisole for 3 consecutive days, twelve were converted to a PPD-positive reaction, whereas out of twenty placebo-treated patients only one became positive at the second test. This difference is significant at the 5% level.
Table 1. Distribution of the patients per tumour site, treatment and extension of the disease in the different studies

<table>
<thead>
<tr>
<th>Primary tumour site</th>
<th>PPD</th>
<th>DNCB</th>
<th>Complement</th>
<th>E-rosettes</th>
<th>Lipofundin</th>
<th>Immunoglobulins</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>P*</td>
<td>L*</td>
<td>P</td>
<td>L†</td>
<td>L‡</td>
<td>P</td>
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<tr>
<td>Colon or rectum</td>
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<td>7</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Stomach</td>
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<td>5</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
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<td>4</td>
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<td>3</td>
<td>1</td>
<td>4</td>
</tr>
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<td>4</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
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<tr>
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<td>1</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
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<td></td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Ovary</td>
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<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Lymphoma</td>
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<td></td>
<td>3</td>
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<td>Leukaemia</td>
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<td>2</td>
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<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
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<td>4</td>
<td></td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Lung</td>
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<td>5</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>9</td>
</tr>
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<td></td>
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</table>

* P = placebo treated controls; L = patients treated with levamisole.
† L = continuous levamisole treatment.
‡ L = intermittent levamisole treatment.
PPD = purified protein derivative.
DNCB = 1-nitro-2,4-dichlorobenzene.

Table 2. Increased skin sensitivity to 1-nitro-2,4-dichlorobenzene after levamisole treatment

<table>
<thead>
<tr>
<th>Primary tumour site</th>
<th>Placebo No. of patients</th>
<th>Placebo No. of tests</th>
<th>Levamisole continuously No. of patients</th>
<th>Levamisole continuously No. of tests</th>
<th>Levamisole discontinuously No. of patients</th>
<th>Levamisole discontinuously No. of tests</th>
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</thead>
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<tr>
<td>Colon or rectum</td>
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<td>29</td>
<td>10</td>
<td>43</td>
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<td>Stomach</td>
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<td>11</td>
<td>2</td>
<td>9</td>
<td>3</td>
<td>12</td>
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<tr>
<td>Pancreas</td>
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<td>4</td>
<td>3</td>
<td>15</td>
<td>1</td>
<td>4</td>
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<tr>
<td>Lung</td>
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<td>3</td>
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<td>1</td>
<td>30</td>
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<td>6</td>
<td>21</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Kidney</td>
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<td>7</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Ovary</td>
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<td>7</td>
<td>3</td>
<td>18</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Lymphoma</td>
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<td>12</td>
<td>3</td>
<td>11</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Leukaemia</td>
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<td>13</td>
<td>4</td>
<td>10</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Larynx</td>
<td>4</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>111</td>
<td>39</td>
<td>158</td>
<td>41</td>
<td>165</td>
</tr>
</tbody>
</table>

Delayed hypersensitivity reaction to dinitrochlorobenzene

After sensitization none of the placebo-treated patients became more DNCB-sensitive than at the first challenge, whereas five out of thirty-nine patients receiving levamisole continuously (P n.s.) and twenty-five out of forty-one patients receiving levamisole intermittently (P<0.00001) became more DNCB-sensitive (Table 2).

Forty-seven cancer patients treated with levamisole continuously or intermittently could be followed-up for more than one year. Of these patients, twenty-eight died, four in a group of sixteen patients who become more DNCB-sensitive during levamisole therapy, and twenty-four in a group of thirty-one patients with no increase in DNCB sensitivity during levamisole therapy (P=0.001).

Lipofundin clearance test

As shown in Fig. 1, the lipid emulsion injected...
in twelve cancer patients was cleared from the blood stream more rapidly after levamisole treatment (mean $T_{1/2} = 2.53$ min) than before treatment (mean $T_{1/2} = 3.18$ min). This activation was significant at the 5% level, suggesting an effect of levamisole on the reticulo-endothelial system.

Although the twelve cancer patients reported here tolerated repeated injections of the lipid emulsion, this test produced, besides other side effects, severe respiratory difficulties in several other patients and is therefore not useful for clinical investigation.

**Serum complement**

As shown in Fig. 2, levamisole significantly elevated serum haemolytic complement activity in cancer patients ($P<0.00001$). The haemolytic complement activity in the control group did not change significantly.

Intergroup differences also were significant ($P<0.00001$).

Serum complement components ($C_3$, $C_4$ and $C_1q$) did not change significantly in the levamisole- or placebo-treated groups.

Interesting is the correlation found between serum haemolytic complement activity and the

**Immunoglobulins**

The immunoglobulins IgA, IgG and IgM did not change significantly after levamisole treatment.

**E-rosette forming cells**

In the placebo group the mean percentage of RFC was 57.2% when first tested and 58.1% in the second test. In the levamisole-treated group the mean percentage of RFC was 56.5% before treatment and 57.2% after treatment. Intra- and intergroup differences were not significant. However, when only

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**Fig. 1.** The effect of levamisole on the half-life ($T_{1/2}$) of a lipid emulsion injected intravenously in twelve cancer patients.

**Fig. 2.** The effect of 1–2 weeks of treatment with levamisole on haemolytic complement activity in sera of cancer patients.
showed recurrence of granulocytopenia in four consecutive days, had to discontinue treatment due to demand. However, rashes with skin reactions (Mackaness, 1976; Levo, Rotter and Ramot, 1975; Rojas et al., 1976). Eilber and Morton (1970) have demonstrated that depressed sensitivity to DNCB in cancer patients is correlated with a bad prognosis and they suggest that tumour growth may be associated with defective cell-mediated immunity. The restoration or enhancement of defective delayed hypersensitivity reactions by levamisole may therefore be of benefit in cancer patients, and this is backed by the finding that patients in whom DNCB sensitivity increased during levamisole treatment had a better prognosis than patients in whom DNCB sensitivity did not change.

The complement system also plays an important role in the defence of the body and it has been previously shown that serum complement activity increases with progressing tumours contributing to the humoral defence of the integrity of the body (Verhaegen et al., 1976). It was observed that levamisole significantly increases haemolytic complement activity in cancer patients, thus potentiating the already enhanced humoral reaction to the tumour.

The lipofundin clearance test measures the phagocytic activity of the reticulo-endothelial system (Lemperlee, Reichelt and Denk, 1971). In cancer patients, levamisole enhanced the clearance of the lipid emulsion and concomitantly increased haemolytic complement activity. The enhancing effects of levamisole on the phagocytic activity of the reticuloendothelial system has also been shown in mice by Hoebeke and Franchi (1973).

The stimulation of the macrophage function by levamisole may explain its effects both on the complement system and on delayed hypersensitivity reactions. Complement components are synthesized by macrophages (Stecher, 1970) and macrophages play an important role in delayed hypersensitivity reactions (Mackaness, 1970; David, 1970; Asherson and Zembala, 1970). The enhancement of delayed hypersensitivity reactions by levamisole may also be due to its interaction with T-lymphocytes, as it was found that levamisole therapy enhances defective E-rosette formation confirming the data from other experiments (Verhaegen et al., 1977a,b; Ramot et al., 1976; De Cock, De Cree and Verhaegen, 1977). Levamisole has also been reported as enhancing lymphoblast transformation (Chan and Simons, 1975; Chan, Lee and Simons, 1976; Lichtenfeld

![Fig. 3. The effect of levamisole treatment on low E-rosette forming cells (RFC) in cancer patients.](image-url)

the patients with low E-rosettes (<50% being 2 standard deviations lower than the mean of eighty-one healthy blood donors) were analysed, levamisole significantly enhanced low rosette formation ($P = 0.01$), whereas in the placebo group RFC remained low (Fig. 3).

**Side effects**

The records of 213 levamisole-treated patients were analysed for possible side effects. In about one third of the patients one of the following complaints was noted: vomiting, tiredness, nausea, diarrhoea, bitter taste, insomnia, headache, dizziness and pruritus. However, the levamisole therapy did not have to be interrupted because of these complaints. Ten patients, receiving 150 mg levamisole for 3 consecutive days every 14 days, showed a generalized skin rash with fever and itching. In five patients, the levamisole therapy was definitely stopped on their demand. In the other five patients, intermittent levamisole treatment was re instituted when symptoms had disappeared and skin rashes did not reappear in four patients. However, the other patient showed recurrence of allergic reactions and developed granulocytopenia each time she took levamisole, but these symptoms disappeared in the levamisole-free periods. In this patient levamisole therapy was withdrawn.

**Discussion**

The above data show that levamisole therapy can restore delayed hypersensitivity reactions to PPD and enhance DNCB sensitivity in some cancer patients, which confirm other previous reports (Tripodi, Parks and Brugmans, 1973; Hirshaut et al., 1973; Levo, Rotter and Ramot, 1975; Rojas et al., 1976). Eilber and Morton (1970) have demonstrated that depressed sensitivity to DNCB in cancer patients is correlated with a bad prognosis and they suggest that tumour growth may be associated with defective cell-mediated immunity. The restoration or enhancement of defective delayed hypersensitivity reactions by levamisole may therefore be of benefit in cancer patients, and this is backed by the finding that patients in whom DNCB sensitivity increased during levamisole treatment had a better prognosis than patients in whom DNCB sensitivity did not change.

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et al., 1976) and the release of lymphocyte mediators (Whitcomb, Merluzzi and Cooperband, 1976; Lieberman and Hsu, 1976; Golding et al., 1976).

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References
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