Levamisole-associated neutropenia and autoimmune granulocytotoxins

S. I. DREW¹, B. M. CARTER¹, D. S. NATHANSON², AND P. I. TERASAKI¹

From the ¹Department of Medicine and Surgery, University of California, and the ²Department of Surgical Oncology, University of California, Los Angeles, California, USA

SUMMARY To investigate possible immune mechanisms responsible for levamisole-associated neutropenia we tested patients with bladder cancer on levamisole therapy. Autoimmune and complement-dependent granulocytotoxic antibodies were detected in 3 patients with levamisole-induced neutropenia. The granulocytopenia appeared to be causally related to the presence of autoantibodies in that pretreatment serum or serum obtained after the restoration of neutrophil counts showed diminished or no granulocytotoxic reactivity. In addition, granulocytotoxins were found in 6 out of 20 (30%) patients receiving levamisole compared to only 2 out of 28 (7.1%) patients on no levamisole or placebo ($P < 0.06$). Hence, screening for granulocytotoxins may forewarn of neutropenia in patients receiving levamisole for a variety of clinical diseases.

Immunological mechanisms underlie certain states of pathological neutropenia (Walford, 1960). With leucoagglutination assays some success has been achieved in the demonstration of humoral autoimmunity to target cell antigens present on granulocytes (Walford, 1960; Lalezari, 1966). However, in the majority of suspected cases of immune neutropenia leucoagglutinins are not found (Pisciotta, 1973).

Agranulocytosis is an uncommon but serious side effect of the use of the immunostimulant drug levamisole (Rosenthal *et al.*, 1977). Immunological mechanisms are suspected to account for the neutropenia, and autoagglutinins have been found in the serum of some affected individuals (Rosenthal *et al.*, 1976). Using a modified complement-dependent microgranulo-cytotoxicity assay (Drew *et al.*, 1977) similar to that employed to serotype lymphocytes (Mittal *et al.*, 1968) we have detected the presence of autoimmune and nonagglutinating granulocytotoxins in 3 patients with severe neutropenia who were receiving levamisole.

Patients and methods

Patient 1. A 65-year-old man with carcinoma of the bladder was allocated by a random method to receive 200 mg/day of levamisole (Jansen R and D,

New Brunswick, New Jersey) on 2 consecutive days per week as part of a double-blind trial of this immunostimulant agent in the treatment of bladder cancer. In December 1977 he developed leucopenia, and the levamisole was discontinued. Bone marrow aspiration showed a marked decrease in mature myeloid elements. Early myeloid precursors, erythroid precursors, and megakaryocytes were well represented. A small drop in peripheral blood lymphocytes accompanied the neutropenia, whereas blood levels of platelets and red blood cells remained normal. Tests for rheumatoid factor and antinuclear antibodies were negative. Serum immunoglobulin levels were normal. The peripheral leucocyte counts gradually returned to normal. To date the patient remains in good health.

Patient 2. A 70-year-old male had a carcinoma of the bladder diagnosed in September 1975. After an initial trial of placebo (Jansen R and D, New Brunswick, New Jersey) the patient was started on levamisole 150 mg/day twice weekly in July 1976. Ten months after initiating the course of levamisole the patient was noted to have a marked decrease in his absolute neutrophil count. The levamisole was stopped and the neutrophil count returned to pre-existing normal levels.

Patient 3. A 52-year-old male with bladder cancer developed agranulocytosis on levamisole. For reasons concerning the patient only 3 sera samples before, during, and after the neutropenic episode were tested against his autologous granulocytes.

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Correspondence to S. I. Drew, MD, Department of Medicine, UCLA School of Medicine, University of California, Los Angeles, California 90024, USA.

SEROLOGY

After the return of the peripheral blood leucocyte counts to normal granulocytes were isolated from freshly drawn heparinised blood as previously described (Drew *et al.*, 1977). Granulocytes obtained from the patients on a single bleeding date were reacted against autologous serum samples that had been collected before, during, and after the course of levamisole therapy and stored frozen at -80°C . One μl of the cell suspension was added to 1 μl of antiserum and incubated at 5°C for 30 minutes. After this period 5 μl of rabbit complement that had been previously absorbed with pooled human red blood cells was added to the cell-antibody mixture and incubation allowed to proceed at 22°C for 3 hours. The percentage of cells killed by a given antiserum was determined by eosin dye exclusion, and the cytotoxicity of a given antiserum against a panel of random granulocytes was expressed as the cytotoxic score (Schocket and Weiner, 1978) and calculated as follows:

$$\text{CS} = \frac{\text{average \% killing of granulocytes by test serum}}{\text{average \% killing of granulocytes by positive control serum}} \times 100$$

A given serum was arbitrarily defined as possessing granulocytotoxic antibodies if the cytotoxic score against a panel of granulocytes exceeded 15. A rabbit antigranulocyte antiserum was the positive control serum and killed 100% of all random cells. Leucoagglutination was performed at 37°C and 5°C with a modification of the method of Jiang and Lalezari (1973) with granulocytes in alcohol-free McCoy's medium containing 1% ethylenediamine tetra-acetic acid.

All the granulocyte preparations tested were enriched to greater than 90% with neutrophils, and the viability of all the cell preparations was greater than 95% of live cells. This did not alter even if granulocytes were suspended in an alcohol-containing solution. In additional control experiments no cytotoxicity was observed if granulocytes were incubated with granulocytotoxins in the presence of heat-inactivated (30 minutes at 56°C) rabbit complement or if granulocytes were incubated with (active) complement in the absence of antibody. The reproducibility of the cytotoxic reactions was previously calculated and found to be 4.5% (error rate percent).

Absorption studies with autologous red blood cells and pooled platelets were carried out with washed cells for 30 minutes on ice. Statistical values were calculated by the Fisher exact test.

Results

Patient 1. A serum obtained from the patient at the time of the neutropenia was cytotoxic for 100% of the autologous granulocytes (Fig. 1), whereas sera obtained prior to the onset of levamisole therapy or after the recovery in the peripheral blood count were non-cytotoxic. This would suggest that a causal relationship existed between the presence of granulocyte autoantibodies and the occurrence of neutropenia. The cytotoxic antiserum produced no autoagglutination with autologous cells. Addition of levamisole to the cell mixture (final concentration 1×10^{-4} mol/l) did not convert the negative cytotoxic reactions to positive scores.

The tissue specificity of the cytotoxic granulocyte antiserum was investigated by direct cytotoxicity and absorption studies. The serum was cytotoxic (CS=70) for a panel of 33 random allogeneic granulocyte preparations. Sera that were negative for the autologous target granulocytes failed to react against the panel cells (CS=0). The endpoint titre of the cytotoxic antiserum against granulocytes

Fig. 1 Patient 1; Percent autologous (□-□) granulocytes killed by the sera of patient 1. Note the absence of leucoagglutination at 5°C and 37°C . The neutrophil counts (▲-▲) appear inversely correlated with the cytotoxic autoreactivity present in the patient's serum

was 1:4. No cytotoxicity was present in any of the patient's sera tested against autologous T lymphocytes. The cytotoxic antiserum did not react with an autologous B-lymphocyte-enriched mononuclear cell population containing 80% B lymphocytes and 20% contaminating monocytes. Pooled platelets or autologous red blood cells at a 1:1 ratio of packed cells to serum were unable to absorb the cytotoxic activity of the antiserum. However, absorption with autologous buffy coat cells at a concentration of 4×10^7 cells/ml of antiserum completely removed the serological reactivity against autologous and random granulocytes.

Patient 2. Sera obtained from patient 2 during the neutropenic episode (Fig. 2) was strongly cytotoxic for his autologous granulocytes and the panel of 33 allogeneic granulocytes (CS=60). The endpoint titre of the cytotoxic antisera against granulocytes was 1:4. A prelevamisole serum and sera obtained within the first 3 months of levamisole therapy were not cytotoxic (CS=0) for autologous granulocytes. None of the cytotoxic granulocyte antisera agglutinated the autologous cell type even in the presence of levamisole.

The cytotoxic antisera were negative for both autologous lymphocytes and a panel of random T and B lymphocytes and were not absorbed with pooled platelets or autologous red blood cells. However, absorption of the granulocytotoxins with autologous granulocytes resulted in a loss of reac-

tivity against the absorbing cell type and a random panel of granulocytes. Fractionation of 1 auto-reactive antiserum on a Sephacryl S-200 Superfine column (Pharmacia, Sweden) yielded a cytotoxic IgM fraction and an inactive IgG fraction. Furthermore, all cytotoxicity could be eliminated by treating the serum with 2-mercaptoethanol (final serum concentration 1.5% 2-ME).

Patient 3. Serum drawn at the time of the neutropenic episode was cytotoxic for 100% of the patient's autologous granulocytes (titre 1:8) and the panel of granulocytes (CS=100), whereas serum obtained before and after the leucopenic event was non-cytotoxic for the panel (CS=0) and autologous granulocytes.

Serum samples from two groups of bladder cancer subjects were tested against the same panel (n=33) of random granulocytes (Fig. 3). Group 1, including the 3 neutropenic cases presented here, comprised 20 persons (18 males and 2 females) receiving 2.5 mg/kg per day of levamisole on 2 consecutive days per week. Six (30%) of the sera were cytotoxic for the panel of granulocytes. In 3 of the patients with demonstrable granulocytotoxins who were receiving levamisole peripheral blood leucocyte levels were within physiological limits, and the cell types were distributed normally. In a second group of 28 persons (15 males and 13 females) receiving no levamisole (n=21) or placebo (n=7) 2 patients (7.1%) had sera that were cytotoxic for

Fig. 2 Percent autologous granulocytes (□) killed by the sera of patient 2. Corresponding leucocyte counts are shown

Fig. 3 Cytotoxic score of granulocytotoxic sera obtained from normal persons and patients with bladder cancer receiving levamisole or placebo

the panel of random granulocytes. Neither subject was neutropenic. From the above findings an association could be demonstrated between levamisole therapy and the presence of granulocytotoxins ($\chi^2=2.90$, $P<0.06$).

Discussion

Autoimmune and cytotoxic granulocyte antibodies were present in the sera of all 3 bladder cancer patients who developed neutropenia while receiving levamisole therapy. The occurrence of granulocyte antibodies appeared to be causally related to the onset of the leucopenia in that sera before and after the neutropenic episode had no granulocytotoxic activity. In comparison with a control group of patients not receiving levamisole granulocyte autoantibodies occurred much more frequently ($P<0.06$) in the sera of patients on levamisole. This finding supports an association between levamisole, neutropenia, and the presence of granulocytotoxic antibodies.

That the granulocytotoxic autoreactivity at the time of the neutropenic episodes is an artefact of the in-vitro assay is very unlikely. This is because less than 5% dead cells were noted in the control

wells with non-cytotoxic sera and control experiments clearly showed the in-vitro cytotoxic reactions to be both antibody-mediated and complement-dependent. The failure of the cytotoxic antisera to agglutinate autologous granulocytes serves to distinguish granulocytotoxic antibodies from autoagglutinins reported in a number of patients with agranulocytosis or levamisole therapy (Rosenthal *et al.*, 1976; Graber *et al.*, 1976; Parkinson *et al.*, 1977). Furthermore, the demonstration of auto-cytotoxicity did not require the presence of levamisole in vitro. The cytotoxic specificity of the granulocyte autoantibodies was restricted to peripheral blood neutrophils, whereas autologous red blood cells, T and B lymphocytes, and monocytes were unreactive (Drew *et al.*, 1978). The spontaneous restoration of peripheral blood neutrophil counts in the 3 cases cited is consistent with our failure to demonstrate granulocyte antigens on colony-forming myeloid stem cells (CFU-C) and early granulocyte precursor cells in preparation.

The role of levamisole in the neutropenic response is at present unknown. We have found that granulocyte antibodies predominated in patients on levamisole and that neutropenia was restricted to patients with granulocytotoxins and receiving levamisole. In addition to promoting antibody production it is possible that levamisole also adversely alters the susceptibility of the target cell to immune sensitisation and subsequent destruction. However, the absence of neutropenia in 3 patients with granulocytotoxins on levamisole therapy (Fig. 3) as well as the occurrence of neutropenia in patients with granulocyte autoantibodies and not receiving levamisole (unpublished) suggests that additional factors as yet undefined play an important supplementary role in the pathogenesis of the autoimmune neutropenia. As we have been unable to detect any immunochemical differences in granulocyte autoantibodies in non-neutropenic subjects versus patients with granulocytopenia, it is our postulate that the occurrence of neutropenia is governed by some inherent defect(s) at the target cell level which promotes the immune destruction of granulocytes. We are now testing this hypothesis.

The greater use of levamisole (Symoens and Rosenthal, 1977) puts a larger population of patients at risk for the development of neutropenia. As the appearance of granulocytotoxins during a course of levamisole therapy may in some cases herald life-threatening neutropenia, screening for these antibodies may opportunely alert the clinician of adverse sequelae in the treated patient population.

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