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Effect of Levamisole on Human Granulopoiesis *in Vitro*[1] (39936)

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Introduction. Granulocyte-macrophage colony formation *in vitro* is dependent upon the presence of a glycoprotein termed colony stimulating factor (CSF) (1, 2). CSF has been derived from a number of sources, including human and mouse serum and urine, human placenta-conditioned medium, peripheral blood monocytes, tissue macrophages, and lymphocytes (3-5). The CSF derived from monocytes, macrophages, placenta-conditioned medium (PCM), and serum is capable of stimulating granulocyte colony formation by human bone marrow cells and may have physiologic significance as a regulator of granulopoiesis. Evidence for this contention is, however, circumstantial at present (6). In addition to stimulating granulocyte colonies, CSF from these sources also stimulates the formation of macrophage colonies *in vitro* and may have physiologic importance in regulation of this cellular system as well (7). The antihelminthic drug, levamisole (L-2,3,5,6-tetrahydro-6-phenyl imadazo (2,1,6)-thiazole hydrochloride), has been shown to affect humoral and cell-mediated immune responses in animals both *in vivo* and *in vitro* (8, 9). Treatment of patients with levamisole has been reported to restore defective cutaneous delayed hypersensitivity and may positively influence the clinical course of some malignancies (10-12). Levamisole also appears to enhance the functional capacity of monocyte-macrophage cells and has been shown to stimulate neutrophil random mobility and chemokinesis (stimulated random migration) (13-15). *In vivo* observations which parallel these *in vitro* findings have also been reported (16, 17). Patients with various types of malignancies who received levamisole in addition to undergoing chemotherapy have been reported to have decreased incidence of infection and hemorrhage, thought to be due to early bone marrow restoration after antineoplastic chemotherpay (18). Animals treated with levamisole, and subsequently challenged with staphylococcal infections, have been reported to have better survival than control animals not receiving the drug (19). Levamisole has been reported to have variable effects on the neutrophil system. In one study the mean neutrophil count was higher in the levamisole-treated group compared to controls, while severe neutropenia has also been reported with the use of this drug (11, 20, 21).

No studies of the effect of levamisole on granulopoiesis *in vitro* have been reported. The following studies were undertaken to determine the effect of levamisole on CSF release by human mononuclear cells *in vitro*. These studies have shown that incubation of human mononuclear cells with low concentrations of levamisole results in increased CSF release, while higher concentrations inhibit CSF release.

Materials and Methods. Separation of leukocytes. Blood was obtained in heparinized tubes from normal human donors, after informed written consent as approved by the Human Subject Research Committee of the University of Colorado Medical Center. The blood was allowed to settle at room temperature and the leukocyte-rich plasma was collected. This was centrifuged at 350*g* for 10 min to obtain a cell pellet. The pellet was suspended in McCoy's 5A medium and layered over a Ficoll-Hypaque gradient (Lymphoprep, Nyegaard and Co., Oslo, Norway) with a density of 1.077. The gradient was centrifuged at 400*g* for 35 min. The mononuclear cell fraction was sepa-

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rated by glass adherence. Mononuclear cells (4×10^6) were incubated in McCoy's 5A medium in 35-mm plastic petri dishes for 2 hr. The petri dishes were washed vigorously with McCoy's 5A medium to remove all nonadherent cells. The adherent population constituted the monocyte fraction, and was generally 25% of the mononuclear cell fraction. The nonadherent population constituted the lymphocyte fraction. Macrophages were obtained by incubating the adherent cell fractions (monocytes), at a concentration of 1×10^6 cells, in McCoy's 5A medium with 20% autologous serum for 3 days at 37° with 100% humidity in an atmosphere of 7.5% CO_2 in air. These cells had the morphologic appearance of macrophages after Wright's staining.

Preparation of conditioned medium. Monocytes, macrophages, or lymphocytes (1×10^6), prepared as above, were incubated in 1-ml aliquots of McCoy's 5A medium with 20% autologous serum in 35-mm petri dishes. Levamisole (2.5 to 1000 ng), in a constant volume of 0.1 ml of McCoy's 5A medium, was added to plates with each cell type, with appropriate control plates containing no levamisole. Plates were then incubated for 48 hr at 37°. The cells were removed by centrifugation and the conditioned medium was stored at 4°.

Assay for CSF. All experiments were done using normal human bone marrow. This was obtained by aspiration into heparinized syringes from the posterior iliac crest of volunteers, after written informed consent as approved by the Human Subject Committee of the University of Colorado Medical Center. The bone marrow aspirate was layered over Ficoll-Hypaque and the mononuclear cell population was obtained as described earlier. Adherent cells were removed from the bone marrow as described by Messner *et al.* (22). Conditioned medium, 0.2 ml, was mixed with 1 ml of McCoy's 5A medium with 15% fetal calf serum and 0.5% agar in 35-mm plastic petri dishes and allowed to gel at room temperature. On top of this, 50,000 nonadherent nucleated human bone marrow cells were layered, mixed with 1 ml of McCoy's 5A medium with 15% fetal calf serum in 0.3% agar. Plates were incubated at 37° with 100% humidity in an atmosphere of 7.5% CO_2 in air. The colony counts were performed at Day 14 with the aid of a dissecting microscope. All experiments were done in triplicate and the mean colony count of the three plates was expressed as the CSF level. Bone marrow when cultured without added CSF produced no colonies. Placenta-conditioned medium (PCM) was used as the source of standard CSF. The method for its preparation is described elsewhere (4). In experiments where the direct effect of levamisole on bone marrow cells was studied, levamisole and the PCM were incorporated in the underlayer.

Results. The effect of levamisole on CSF release by peripheral blood monocytes is shown in Fig. 1. At low concentrations (2.5 to 5.0 ng/ml), levamisole significantly enhanced CSF release by monocytes ($P < 0.01$). At higher concentrations no significant increase in CSF release was found; indeed, inhibition was observed with concentrations greater than 25 ng/ml. CSF release by peripheral blood lymphocytes was not affected by levamisole, as shown in Fig. 1.

The effect of levamisole on CSF release

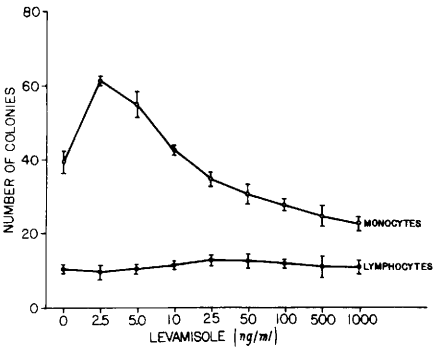

FIG. 1. The effect of increasing concentrations of levamisole on colony stimulating factor (CSF) release by monocytes (open circles) and lymphocytes (closed circles). Forty-eight-hour conditoned medium from control monocyte cultures (without added levamisole) produced 40 colonies. Addition of 2.5 and 5.0 ng/ml of levamisole significantly enhanced CSF release ($P < 0.01$). Levamisole concentrations greater than 25 ng/ml produced inhibition of CSF release by monocytes. CSF release by lymphocytes was not affected by levamisole. Each point on the curve is the mean colony count of three plates. The bars represent the standard error of the mean (SEM).

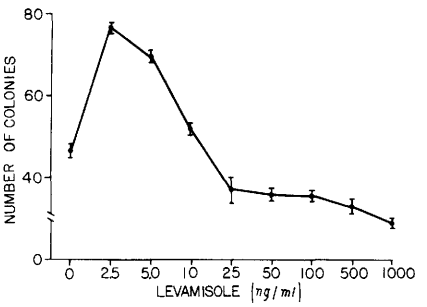

FIG. 2. The effect of increasing concentrations of levamisole on colony stimulating factor (CSF) release by macrophages. Forty-eight-hour conditioned medium from control cultures (no levamisole) produced 47 colonies. Addition of 2.5 and 5.0 ng/ml of levamisole significantly enhanced CSF release ($P < 0.01$). Concentrations above 25 ng/ml were inhibitory. Each point on the curve is the mean colony count of three plates. The bars represent the standard error of the mean (SEM).

by human macrophages was similar to its effect on CSF release by monocytes. As demonstrated in Fig. 2, low concentrations of levamisole were stimulatory ($P < 0.01$) while higher concentrations (25 ng/ml and above) were inhibitory.

To determine if the stimulatory effect of levamisole was due to a direct effect of the drug on CSF release by monocytes and macrophages, or mediated either by a direct stimulatory effect on the bone marrow or as a result of increased bone marrow responsiveness to CSF, the following experiment was performed. A standard preparation of CSF derived from the human placenta (at a suboptimal concentration of 1:4 dilution) was mixed with various concentrations of levamisole, ranging from 2.5 to 1000 ng/ml, and incorporated in the underlayer. The results of this experiment are shown in Fig. 3, and demonstrate that levamisole did not stimulate bone marrow directly, nor enhance its responsiveness to CSF (PCM) at any concentration. It should also be noted that even high concentrations of levamisole (1 μg/ml) did not inhibit bone marrow directly. These data suggest that the inhibitory effect of high concentrations of levamisole was not a direct effect on the bone marrow, but a result of decreased CSF release by monocytes and macrophages. To further explore the direct effect of levamisole on bone marrow, or responsiveness of bone marrow to CSF, doubling dilutions of a standard CSF (PCM) were mixed with a concentration of levamisole known to be stimulatory (2.5 ng/ml). This levamisole-CSF mixture was incorporated in the underlayer. No significant stimulation was noted (Fig. 4), suggesting that levamisole does not affect the target cells directly or by modulating its responsiveness to CSF.

To investigate the effect of levamisole on preformed CSF, levamisole in high concentrations (1 and 10 μg/ml) was incubated with CSF derived from monocyte-conditioned medium. After 24 hr of incubation, CSF activity was determined before and after dialysis. Samples (3 ml) of the conditioned medium were dialyzed at 4° in visking tape against distilled water for 72 hr. This maneuver removes all of the drug, but does not remove CSF. As shown in Fig. 5, CSF activity was not changed by incubation with levamisole, and even very high concentrations of levamisole did not inhibit bone marrow target cells directly. These data further indicate that the inhibitory effect of high-dose levamisole in monocyte-macrophage cultures was the result of an effect on CSF release.

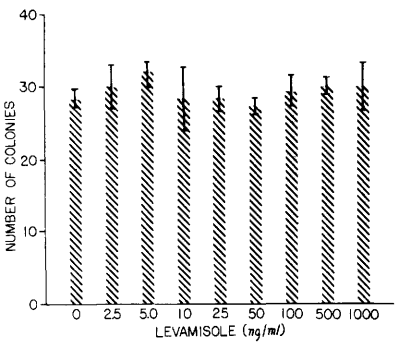

FIG. 3. The effect of various concentrations of levamisole on the colony forming capacity of normal human bone marrow stimulated by a standard preparation of colony stimulating factor (CSF) derived from human placenta. Levamisole in increasing concentrations was incorporated in the underlayer with the CSF. Normal human bone marrow at a concentration of 50,000 nucleated cells/ml was suspended in the overlayer. Levamisole did not stimulate bone marrow directly nor enhance its response to CSF at any concentration. Each hatched bar on the graph represents the mean colony count of three plates. The standard error of the mean (SEM) is also shown.

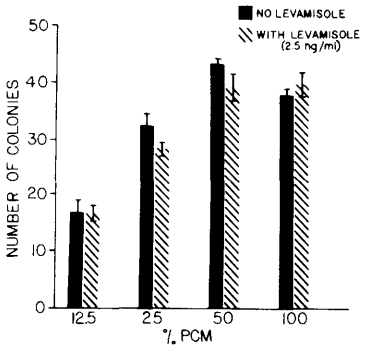

FIG. 4. The effect of a stimulatory concentration of levamisole on the colony forming ability of normal bone marrow in the presence of doubling dilutions of a standard source of colony stimulating factor (CSF) derived from human placenta. A stimulatory concentration of levamisole (2.5 ng/ml) and doubling dilutions of a standard source of CSF were incorporated in the underlayer. Normal human bone marrow was used as the target cell. No significant stimulation at any concentration of CSF was noted. The solid bars represent controls (i.e., no levamisole added to the plate), while the hatched bars represent plates with levamisole. Each bar is the mean colony count of three plates. The standard error of the mean (SEM) is also shown.

Discussion. It has been clearly demonstrated that the monocyte-macrophage cellular system is the predominant source of CSF in humans (23-27). Enhancement of CSF release by these cells can be produced by incubation with various bacterial and nonbacterial materials—endotoxin, gram-positive bacteria and their products, and Poly I·Poly C (28-30). The results presented here indicate that incubation of human monocytes and macrophages with levamisole at low concentrations also results in increased CSF release, while higher concentrations appear to be inhibitory. These results could also be explained by the effect of levamisole on the production of an inhibitor of granulopoiesis: Low doses decrease inhibitor production, while high doses stimulate production of this factor. This phenomenon is under investigation in our laboratory.

Levamisole has been shown to stimulate the immune system *in vivo* and *in vitro* at low doses and may produce immunosuppression in higher doses (8). The data presented here suggest that this is not confined to the classic immune system and may also be operative on the granulocyte system as well.

Levamisole has been noted previously to have a stimulatory effect on monocyte-macrophage function resulting in improved phagocytosis and killing of bacteria (13, 14). The present findings of increased CSF release and granulocyte colony formation are further evidence of the stimulatory effect of levamisole on the monocyte-macrophage system. The protective effect of levamisole in infected animals may be related in part to a stimulatory effect of this drug on CSF production or release by the monocyte-macrophage complex resulting in increased granulopoiesis and availability of greater numbers of granulocytes for localization and eradication of bacteria. It is of interest and importance that, in some studies, enhanced monocyte function has not been observed (31), and this may be a dose-related phenomenon, as demonstrated in the present studies.

Lods *et al.* have suggested that levamisole

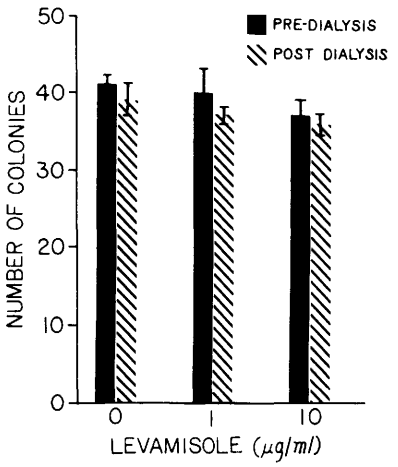

FIG. 5. The effect of levamisole on preformed colony stimulating factor (CSF) derived from monocyte-conditioned medium. CSF was incubated with levamisole at concentrations of 1 and 10 μg/ml for 24 hr. After this incubation, CSF activity was determined before and after dialysis. CSF activity was not changed by incubation with levamisole, and even high concentrations of levamisole did not inhibit bone marrow colony growth. The solid bars represent predialysis values, while the hatched bars show the colony counts after dialysis. Each bar is the mean colony count of three plates. The standard error of the mean (SEM) is also shown.

may have a direct protective effect on pluripotential stem cells, as suggested by the low incidence of infection and hemorrhage in their patients receiving this drug in conjunction with antineoplastic chemotherapy (18). The present studies do not indicate that levamisole has any effect on committed stem cells *in vitro,* but, since this system does not measure the kinetics of earlier uncommitted stem cells, this point deserves further investigation.

The present studies also indicate that levamisole does not enhance CSF release by lymphocytes. This is in agreement with previous studies that levamisole does not stimulate lymphocyte function *in vitro* (32, 33). Though levamisole can increase lymphokine release (34), the regulation of CSF and lymphokine release seems to be under different control mechanisms (35).

Further work is necessary before attempting to extrapolate the present findings to clinical situations. The noted stimulatory effect of low concentrations of levamisole on granulocyte production *in vitro* suggests, however, that this drug may be beneficial in patients with decreased granulocyte production or increased granulocyte demand.

Summary. The effect of levamisole, a potent immunomodulator, was studied on granulopoiesis *in vitro*. Low concentrations of levamisole stimulated monocytes and macrophages and resulted in enhanced colony stimulating factor (CSF) release. Higher concentrations of levamisole were inhibitory to CSF release. Levamisole did not affect bone marrow directly to produce stimulation or inhibition of granulopoiesis. Levamisole did not degrade or potentiate preformed CSF. Improved survival of infected animals may in part be related to increased granulopoiesis. The dose-related effect of levamisole on CSF release by the monocyte–macrophage system may be responsible for the variable effect on the neutrophil system noted *in vivo*.

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