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The *in vivo* and *in vitro* Effects of Levamisole in Patients with Lepromatous Leprosy[1]

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Leprosy is divided on immunological and clinical grounds into two major polar types: tuberculoid, which has a normal intact cell-mediated immune response, and lepromatous, which manifests a severe deficiency of cell-mediated immunity (CMI). This abnormality is related to defects in both function of T-lymphocytes ([2,7,9,25,28]) and their numbers in the peripheral blood and lymph nodes ([5,10,12,22,26]). Abnormalities of neutrophil function, mainly chemotaxis, have also been reported ([23,28,29]).

Levamisole, a broad-spectrum antihelminthic, influences host defenses by modulating cell-mediated immune responses both *in vivo* and *in vitro*. It restores T-cell functions such as E-rosette formation, cell-mediated cytotoxicity, lymphokine production and nucleic acid or protein synthesis ([4,14,24,27,30,31]). The effects are most marked in hypofunctional cells from compromised hosts and seldom are cells from normal persons affected ([24]). Levamisole has been shown to alter the action of cyclic nucleotides by increasing cGMP levels and decreasing cAMP levels ([8]).

Occasional reports have appeared concerning the effect of levamisole in lepromatous leprosy, both in its capacity to enhance the cell-mediated immune responses and to alter the number of acid fast bacilli (AFB) ([11,13,21]).

Because of the paucity of data concerning the effect of levamisole in leprosy and its well known effect as an immunomodulator of CMI in anergic states, we undertook the following studies before and during levamisole therapy: delayed hypersensitivity skin-reactions using a variety of antigens and lepromin, skin biopsies, lymphocyte transformation and production of leukocyte inhibition factor (LIF) following phytohemagglutinin (PHA) stimulation and percentages of E and EAC rosettes. Absolute numbers of leukocytes were also monitored during the course of levamisole therapy.

MATERIALS AND METHODS

Patients. Patients were residents of Westfort Leprosarium, situated 40 miles from the laboratory of investigation, and were classified into five groups, true tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL) and true lepromatous (LL), according to Ridley and Jopling ([18]) and on clinical grounds. For the purpose of this study patients were divided into two main groups, tuberculoid, which consisted mainly of BT cases, and a lepromatous group, which consisted of both borderline lepromatous (21 cases) and true lepromatous (19 cases). Tuberculoid patients served as controls in the evaluation of *in vitro* cell-mediated immunity.

Patients with lepromatous leprosy were divided into the following two groups:

Group I. This group comprised twenty patients who had been admitted to Westfort prior to the commencement of this trial and were all receiving specific antileprosy treatment which consisted of a six week course of rifampin and long term dapsone. Half the number of patients received a placebo (glucose coated tablets) and the other half levamisole, 150 mg on 2 consecutive days weekly, for 6 weeks. Patients receiving the placebo consisted of seven females (4 LL

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and 3 BL) and three males (2 LL and 1 BL) and those receiving levamisole consisted of four females (3 LL and 1 BL) and six males (2 LL and 4 BL). Patients in Group I were subjected only to delayed hypersensitivity skin testing with various antigens. All the patients in this group continued with their antileprosy treatment throughout the trial. The mean age (years) and range for the female patients was 36.7 (15–60) and for the males 33.4 (20–56).

Group II. Group II consisted of twenty patients who were newly admitted to Westfort and had not received any specific antileprosy treatment. All the patients in this group received levamisole, 150 mg, on 2 consecutive days weekly, for 6 weeks. No antileprosy treatment was administered during the duration of the trial. Patients comprising this group were fifteen males (5 LL and 10 BL) and five females (3 LL and 2 BL).

The following tests were conducted on patients in this group : delayed hypersensitivity skin reactions with various antigens, skin biopsies, lymphocyte transformation and production of leukocyte inhibition factor and finally, the percentage of E and EAC rosettes. These tests were performed on admission and repeated during the last week of the trial. The mean age (years) and range in this group was females 29.2 (19–40) and males 36.9 (24–50).

Lymphocyte separation. Blood was obtained by venipuncture and mixed with preservative free heparin (Panheparin, Abbott, 10 units per ml). Cells were separated by centrifugation on a Ficoll-Hypaque gradient. Mononuclear cells (MN) were harvested from the plasma-Ficoll interphase, washed three times in minimum essential medium (MEM) and adjusted to a concentration required for the different tests.

Lymphocyte transformation. Lymphocytes were made up to a concentration of 2×10^6/ml in MEM (buffered with hepes, pH 7.2–7.4) containing 10% heat inactivated (56°C for 30 minutes) autologous serum, 2 mM l-glutamine (Flow Laboratories), 100 units/ml penicillin and 100 μg/ml streptomycin (Glaxo-Allenbury). Cells were cultured in round-bottom microtiter plates (2×10^5 cells/well). When PHA (Reagent grade, Burroughs Wellcome 10 μg/ml) was used to stimulate the cells, incubation was carried out for 72 hr at 37°C in 5% CO_2 in humidified air. The cultures were pulsed with methyl ^{3}H-thymidine, 0.2 μCi/well (specific activity 17 Ci/mmol, Radio-Chemical Centre, Amersham, England) for 18 hr before harvesting with a multiple automatic sample harvester (Mash II) and the thymidine incorporation assessed by liquid scintillation.

The results were expressed as increments in thymidine incorporation (experimental [E] minus control [C] counts per minute [cpm]).

Lymphokine production. Lymphocytes (2×10^6/ml) were incubated in the presence (active supernatant) and in the absence (control supernatant) of 10 μg/ml PHA in plastic tissue culture tubes (Falcon) for 2 hr at 37°C. The cells of both cultures were washed three times in MEM and then resuspended to their original volume in hepes buffered MEM plus 10% inactivated autologous serum. Cultures were incubated at 37°C in 5% CO_2 for 72 hr. The cell-free supernatants from the unstimulated and PHA-pulsed cultures were obtained by centrifugation at $250 \times g$ for 10 min. The supernatants were stored at −20°C until assayed for lymphokine production.

LIF assay. LIF was assayed using the modified agarose gel technique of Clausen ([3]). After 18–20 hr incubation in 5% CO_2, migration areas were fixed in methanol and 35% formaldehyde for 30 min; the agarose was removed and the migration areas stained with 0.5% crystal violet. The migration pattern was projected and traced and the area of migration was measured by planimetry. The migration index (MI) was calculated as follows:

$$\text{MI} = \frac{\text{Area of migration in presence of active supernatants}}{\text{Area of migration in presence of control supernatants}}$$

E and EAC rosettes. Erythrocyte (E) rosettes and erythrocyte antibody complement (EAC) rosettes were prepared as described previously by Sher, *et al.* ([22]). Preparations were performed in duplicate, 200 lymphocytes being counted and the number of lymphocytes forming rosettes (three or more sheep red blood cells [SRBC] per lymphocyte) was expressed as a percentage.

Delayed hypersensitivity skin testing. The following antigens were used: *Candida albicans* 1/100 (Hollister-Stier Laboratories), Streptokinase-streptodornase (SKSD) 1:4 units (Lederle Laboratories) and purified protein derivative (PPD); 0.1 ml of each antigen was injected intracutaneously in the volar aspect of the forearm and the reaction was read 48 hr later. An indurated reaction of 5 mm or greater was recorded as positive.

Lepromin test. Lepromin was prepared from freshly biopsied human tissue according to the method of Rees (personal communication). Briefly, biopsied material was collected under sterile conditions. After the epidermis and fatty tissue were removed, the remaining tissue was thoroughly homogenized in 4 ml of 1% bovine albumin (Armour Pharmaceutical) in physiological saline. This homogenate was transferred to a 15 ml conical centrifuge tube, centrifuged at 600 × *g* for 5 min and the supernatant removed and centrifuged at 2000 × *g* for 30 min. Residual lipid was removed with a Pasteur pipette. The pellet was then resuspended in 1% albumin saline solution and the mycobacteria were counted and suspended to a final concentration of 6×10^6/ml. This preparation (0.1 ml) was injected intracutaneously for the lepromin test and positive results were always obtained with patients diagnosed both clinically and by biopsy as having the tuberculoid type of leprosy.

Skin biopsies. Skin biopsies were performed before treatment and repeated during the last week of levamisole therapy. Both biopsies were taken from similar areas for histology and assessment of *M. leprae* numbers ([19]).

Statistical analysis. All data were statistically assessed by the Student's *t*-test except when stated otherwise. Due to unavoidable circumstances, such as patients absconding during the trial, not all patients were fully tested.

RESULTS

Delayed hypersensitivity skin reactions. The lepromin reactions in Groups I and II were all negative prior to treatment with levamisole and remained unchanged following levamisole administration. In Group I, using PPD antigen, one patient on placebo and one on levamisole converted from a negative reaction to positivity. Using SKSD one patient on placebo and four on levamisole converted to a positive reaction. With candida antigen two patients on placebo and one on levamisole converted from negative to positive. No significant difference was found between those patients on placebo and those receiving levamisole (Fisher exact test). In Group II, in which all patients were treated with levamisole, one patient tested with PPD and one with candida converted from negative to positive and four patients receiving SKSD showed conversion. The number of patients in Group II was too small for adequate statistical analysis. The remaining patients in both groups showed no conversion.

TABLE 1. *Blastogenic response of untreated leprosy mononuclear cells to PHA (cpm).*[a]

	Before treatment with levamisole Mean ± S.E.M.	After treatment with levamisole Mean ± S.E.M.
Lepromatous (N = 17)	22,945 ± 4391	16,296 ± 2801
Tuberculoid[b] (N = 17)	35,223 ± 4919	
Normal controls (N = 15)	33,738 ± 8107	

There was no statistical difference in lepromatous cases before and after treatment with levamisole. A significant statistical difference was found between lepromatous and tuberculoid cases both before and after treatment of lepromatous patients with levamisole ($p < 0.05$). There was no significant difference between tuberculoid cases and normal volunteers.

[a] Results were expressed as increment in ^{3}H-thymidine incorporation (experimental [E]—control [C]—counts per minute).

[b] Patients with tuberculoid leprosy were not treated with levamisole.

Lymphocyte transformation. The results of lymphocyte transformation following PHA stimulation as measured by ^{3}H-thymidine incorporation is shown in Table 1. There was a statistical difference ($p < 0.05$) between lepromatous and tuberculoid patients both before and after levamisole in Group II. However, there was no significant increase in ^{3}H-thymidine uptake in lepromatous patients before and during lev-

TABLE 2. *Production of LIF by lepromatous and tuberculoid mononuclear cells following PHA stimulation (Migration Index).*[a]

	Before treatment with levamisole Mean ± S.E.M.	After treatment with levamisole Mean ± S.E.M.
Lepromatous (N = 19)	0.89 ± 0.03	0.91 ± 0.03
Tuberculoid (N = 13)	0.76 ± 0.04	
Normal controls (N = 15)	0.69 ± 0.08	

[a] Migration inhibition of 0.80 or less is considered significant in this laboratory.

amisole administration. Results were expressed as the increment in ^{3}H-thymidine incorporated (experimental value—control).

LIF production. Whereas the mean Migration Index for the tuberculoid group was 0.76 ± 0.04, for the lepromatous group it was 0.90 ± 0.03 ($p < 0.05$). Although four patients with lepromatous leprosy showed some LIF production during levamisole administration which was not produced prior to levamisole, there was no statistically significant increase in LIF production in this group of patients during levamisole treatment (Table 2). There was no correlation between E and EAC rosette formation and LIF production in these four cases. However, all four of these patients were borderline lepromatous cases.

E and EAC rosettes. Lymphocytes from lepromatous leprosy patients (Group II) showed decreased numbers of E and EAC rosettes prior to levamisole therapy. This deficiency was corrected following the administration of levamisole (The Figure and Table 3). This increase was statistically significant for E rosettes ($p < 0.05$). Levamisole did not, however, increase the number of E and EAC rosettes when these were within the normal range (The Figure). A statistical difference in E and EAC rosettes between tuberculoid patients and lepromatous patients prior to levamisole was also found ($p < 0.01$ and $p < 0.01$, respectively).

TABLE 3. *Absolute lymphocyte counts and percentages of E and EAC rosettes before and after levamisole administration.*

Patient	Diagnosis	Before levamisole			After levamisole		
		Total lymphocyte count	% E rosettes	% EAC rosettes	Total lymphocyte count	% E rosettes	% EAC rosettes
1	BL	2336	46	26.5	3266	60	25
2	BL	3354	57	21.5	2970	68.5	20
3	BL	2337	49.5	7.5	2108	51	17.5
4	LL	990	56	21.5	1344	ND	ND
5	LL	1035	65	27.5	1188	63	23
6	BL	1332	51	8.5	3400	75	13
7	BL	592	49	12.5	1085	52	25
8	LL	2538	61	11	2652	66.5	21
9	BL	2160	49	25	609	54	24
10	LL	1665	50	8	1710	68	23
11	BL	1612	44.5	7	3025	64.5	20
12	LL	2470	59.5	10.5	2964	50.5	20.5
13	BL	2542	45	22.5	2730	30	21
14	LL	1624	54.5	7.5	3110	63	17
15	LL	2244	46	10.5	2074	59	23
16	BL	2183	45	26.5	3680	64	28.5
17	BL	4308	43.5	13.5	2700	ND	ND
18	LL	2184	50	7.5	2905	55	21
19	BL	1581	57	2.5	2100	68	21
20	LL	1519	32	5.5	1920	64	13
Mean ± S.E.M.		2030 ± 189	50.5 ± 1.7	15 ± 1.8	2377 ± 192	60 ± 2.4	21 ± 0.9

BL = borderline lepromatous, LL = true lepromatous, ND = not done.

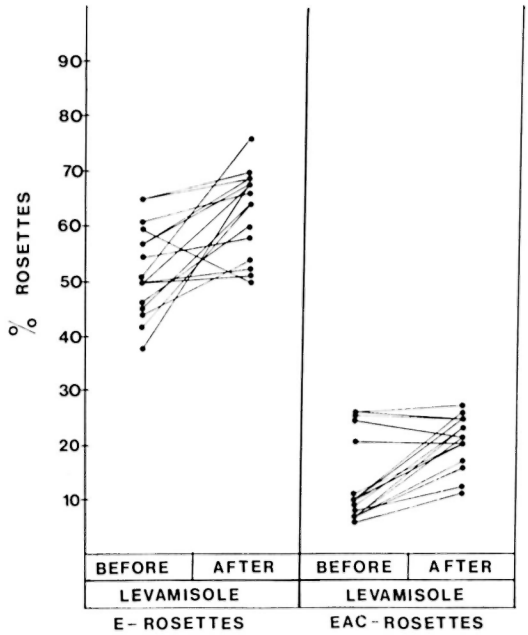

FIGURE. E and EAC rosettes from lepromatous leprosy patients before and after levamisole therapy. Mean ± S.D. for normals, E Rosettes: 65.4 ± 7.3, EAC rosettes: 21.7 ± 4.7%.

Skin biopsy. Skin biopsies from patients of both groups remained essentially unchanged in regard to the histology and number of *M. leprae*.

Leukocyte profile. Neutropenia was not observed in any patient receiving levamisole. It would seem that levamisole had no significant effect on the absolute neutrophil counts compared to the placebo. Absolute lymphocyte counts in Group I patients showed no significant differences. In Group II, however, there was a statistically significant increase in the absolute lymphocyte count; 13 of 20 patients showed an increased count of greater than 10% during the final week of levamisole therapy, four were unchanged, and three decreased ($p = .018$ Wilcoxan signed rank test). Table 3 shows the absolute lymphocyte counts and percentages of E and EAC rosettes before and after levamisole administration in individual patients in Group II.

DISCUSSION

Because of the immunomodulatory effect of levamisole on defective T lymphocyte function and the depressed CMI in lepromatous leprosy, it was justifiable to expect that levamisole might exert a beneficial effect in this type of leprosy.

During this six week trial of levamisole in both previously treated and untreated patients with lepromatous leprosy no clinical improvement or untoward reactions were observed. There is no published data to show clinical improvement in patients with lepromatous leprosy who were treated with levamisole alone. Martinez and Zaias ([11]) showed that when levamisole was used as an adjunct to dapsone, there was a decrease in clinical lesions and incidence of reactions compared to a placebo. However, the trial involved a small number of cases and the dose of levamisole was only 150 mg every two weeks. Since no obvious clinical improvement was observed in the Group II patients after 6 weeks in the present study, we felt unjustified in withholding specific antileprosy treatment any longer.

Our observations revealed the following improvements in cellular immunity: increase in the number of peripheral circulating E and EAC rosetting lymphocytes and production of leukocyte inhibition factor in a small number of cases (four) previously negative. The negative aspects of the trial were: no conversion of the lepromin reaction and insignificant conversions of candida and PPD skin reactions, although a small number of SKSD conversions from negative to positive were noted. No improvements in the skin biopsies were found, nor was there any augmentation of lymphocyte transformation following PHA stimulation.

Several investigators have shown increased numbers of E rosettes following levamisole therapy in conditions in which these cells are decreased ([16,24,27]). Levamisole has also been shown to increase the number of E rosetting cells, when incubated *in vitro*, where these cells are decreased ([14,24,31]). We have shown a statistically significant increase in numbers of E rosetting lymphocytes following levamisole administration. Although Rosenthal, *et al.* ([20]) failed to show an increase in E rosetting cells following levamisole therapy, this effect has been found by most other investigators.

Recent evidence suggests that the restoration of numbers of E rosettes in Hodg-

kin's disease may be due to the removal of blocking factors of receptors for SRBC on T lymphocytes by levamisole. Ramot, *et al.* ([17]) have shown ferritin to be such a blocking factor in Hodgkin's disease. Whether this is true for lepromatous leprosy remains to be determined. If not ferritin, then certainly other blocking factors may be involved. The literature concerning the effect of levamisole on EAC rosettes is scanty. Wybran and Govaerts ([31]) showed a significant decrease of EAC rosettes when levamisole was added to normal lymphocytes *in vitro* and Bensa, *et al.* ([1]) reported a return to normal of elevated B cell numbers in a case of angioimmunoblastic lymphadenopathy. In another case levamisole failed to increase the numbers of B cells previously decreased by immunosuppression ([6]). Ramot, *et al.* ([16]) showed that in Hodgkin's disease the immunoglobulin receptor-bearing cell number declined only when the incubation with levamisole affected the number of E rosette-forming cells. Our results revealed no increase in number of EAC rosetting cells when these numbers were within the normal range. Rosenthal, *et al.* ([20]) found a slight increase in B cells after levamisole in patients with rheumatoid arthritis and ankylosing spondylitis. However, the numbers were not depressed prior to levamisole and the increase was not statistically significant. The discrepancy in our findings of the stimulatory effect of levamisole on lymphocyte numbers between Groups I and II is difficult to explain. One possible explanation is that the antileprosy drugs, administered to the patients in Group I, may have been responsible for the decrease in lymphocyte numbers negating the effect of levamisole. A further possibility is the fact that patients in Group I had been on antileprosy treatment for varying periods of time. Sher, *et al.* ([22]) have shown increased absolute lymphocyte counts following specific treatment.

There was no increase in lymphocyte proliferation following levamisole therapy and, although four patients showed LIF production following levamisole, most cases remained unchanged. Symoens and Rosenthal ([24]) in a review of the effect of levamisole in augmenting CMI point out that levamisole may increase lymphokine production, protein, and nucleic acid synthesis and that the functional state of lymphocytes, the concentration of both antigen and levamisole, time of levamisole application, and nature of the underlying disease state were factors which influenced the effect of levamisole, which was per se unable to augment CMI.

No untoward side effects or reactions were experienced in both groups during the period of levamisole administration. The well-known complication of agranulocytosis was not found. In conclusion, our study was unable to demonstrate conclusively any augmentation of CMI, although the numbers of E and EAC rosetting cells were increased.

SUMMARY

Levamisole, 150 mg daily, was administered on 2 consecutive days per week for 6 weeks to two groups of patients with lepromatous leprosy. Group I was composed of patients who were receiving specific antileprosy therapy for varying periods of time and Group II were untreated lepromatous patients. Whereas half the patients in Group I received levamisole and the other half a placebo, those in Group II all received levamisole. Patients in both groups showed a) no clinical improvement, b) no conversion of the lepromin reaction, c) no histological change in skin biopsies, d) conversion of SKSD skin reactions from negative to positive in 20% of patients from each group, and e) unaltered absolute neutrophil counts. Whereas the total lymphocyte counts were unchanged in patients from Group I, 13 patients from Group II showed an increased lymphocyte count of greater than 10%. Lymphocyte transformation and lymphokine production in the second group showed no significant change, although four patients showed some lymphokine production after levamisole therapy. E and EAC rosettes were significantly increased in cases where these were reduced prior to treatment with levamisole. Side effects due to levamisole were not experienced.

RESUMEN

Se trató a dos grupos de pacientes con lepra lepromatosa con 150 mg de levamisol administrados en 2 días sucesivos de cada semana, durante 6 semanas. El

grupo I estuvo compuesto por pacientes que habían estado recibiendo una terapia antileprosa especifica por periodos variables de tiempo y el grupo II, por pacientes sin tratamiento. Mientras que sólo la mitad de los pacientes en el grupo I recibieron levamisol, y la otra mitad placebo, todos los pacientes del grupo II recibieron levamisol. Los pacientes de ambos grupos mostraron: a) ninguna mejoría clínica, b) no conversión de la reacción a la lepromina, c) ningún cambio histológico en las biopsias de piel, d) conversión de la reactividad en piel al antígeno SK-SD de negativa a positiva en el 20% de los pacientes de cada grupo, y e) no alteración en las cuentas totales de neutrófilos. Mientras que las cuentas totales de linfocitos en los pacientes del grupo I permanecieron sin cambios, 13 pacientes del grupo II mostraron incrementos mayores del 10% en sus cuentas de linfocitos. La transformación de linfocitos y la producción de linfocinas en el segundo grupo no mostraron cambios significantes aunque cuatro de los pacientes presentaron una cierta producción de linfocinas después del tratamiento con levamisol. Las rosetas E y EAC estuvieron significativamente incrementadas en los casos donde sus niveles estuvieron reducidos antes de iniciarse el tratamiento con levamisol. No se observaron efectos colaterales debidos al levamisol.

RÉSUMÉ

On a administré à deux groupes atteints de lèpre lépromateuse, pendant 2 jours consécutifs par semaine, du lévamisole à la dose de 150 mg par jour, et ceci pendant 6 semaines. Le Groupe I était composé de malades recevant une thérapeutique antilépreuse spécifique pendant des périodes de temps diverses, alors que le Groupe II était composé de malades lépromateux non traités. Alors que la moitié des malades du Groupe I a reçu du lévamisole et l'autre moitié un placebo, ceux du Groupe II recevaient tous du lévamisole uniquement. Les observations relevées dans les malades de l'un et l'autre groupes, sont les suivantes: a) aucune amélioration clinique; b) aucun virage de la réaction lépromínique; c) aucune modification histologique dans les biopsies cutanées; d) virage des réactions cutanées SK-SD de négatif à positif chez 20% des malades de chaque groupe; e) numérations sans changement du nombre absolu de neutrophiles. Alors que les numérations du nombre total de lymphocytes restaient inchangées chez les malades du Groupe I, 13 malades du Groupe II ont montré un accroissement du nombre de lymphocytes supérieur à 10%. La transformation lymphocytaire, de même que la production de lymphokine, n'a montré aucune modification dans le deuxième groupe, alors que 4 malades du premier groupe ont témoigné d'une production de lymphokine après thérapeutique par levamisole. Les rosettes E et EAC étaient significativement augmentées dans les cas où elles étaient réduites avant l'instauration du traitement par le lévamisole. Aucun effet secondaire dû au lévamisole n'a été enregistré.

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