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IN VITRO PARAMETERS OF
CELL-MEDIATED IMMUNE REACTIONS
IN HEALTHY INDIVIDUALS
FOLLOWING IMMUNE-STIMULATION
ATTEMPTS WITH LEVAMISOLE

By

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Levamisole has been used for several years as an anthelmintic in man and has proved to be safe and effective (14). Recently, it has been shown that levamisole, when given orally, influenced phagocytosis of peritoneal macrophages *in vitro* (13), and increased metabolic activity of macrophages from rats and mice (7). Levamisole has been found to increase cell-mediated immunity, when this was deficient or depressed (15). Levamisole administration reduced the incidence of both primary tumours and lung metastases in the Lewis lung tumour system in mice (12), while Potter et al. (11) reported that levamisole was found inactive in the treatment of four other experimentally induced tumours. In some studies an effect of levamisole on antibody production has been shown, but the results are inconsistent (2, 11).

In order to see if levamisole could influence a normal immune reactivity, we investigated some *in vitro* parameters of cell-mediated immune reactions in healthy persons during administration of levamisole. The following *in vitro* investigations were done: leucocyte migration test (LMT), with

purified protein derivative (PPD); lymphocyte transformation test (LTT) with PPD, phytohaemagglutinin (PHA) and poke weed mitogen (PWM); and estimation of ATP-ase activity of peripheral blood lymphocytes.

MATERIAL AND METHODS

The study includes 10 healthy volunteers, 5 males and 5 females, all under the age of 32 years and all known to be mantoux positive. A total of 150 mg levamisole was given orally to each person on day 1, 2 and 3. This was repeated on day 7, 8 and 10. Blood samples were drawn on day 1, 3 and 10 for estimation of leucocyte migration test on agarose, lymphocyte transformation tests and lymphocyte ATP-ase activity.

Leucocyte Migration Test (LMT)

The leucocyte migration test on agarose in the present study was performed in accordance with the technique of Clausen (4). The gel medium was composed of 1.5 per cent indubiose agarose (l'Industrie Biologique Francaise S.A.) in TC medium 199 (Difco), with 10 per cent horse serum (Statens Serum Institut, Copenhagen), penicillin 66 Units per ml and streptomycin 66 μ g per ml. HEPES buffer was added and pH adjusted to 7.3. The medium was poured into disposable Petri dishes, and wells with a diameter of 2.3 mm were punched. Venous blood was collected in 10 ml polystyrene tubes containing 250 IU heparin. After sedimentation for 1 h at 37° C, the leucocyte-rich plasma was transferred to thrombotest tubes and spun at 220 g for 5 min. The pellets were washed three times in Hanks balanced salt solution. The leucocytes were resuspended in TC 199, tissue culture medium containing 10 per cent horse serum, penicillin, streptomycin and HEPES buffer, as mentioned above. The pH was adjusted to 7.3 with 0.1 NaOH. PPD human (PPD, RT 32, Statens Serum Institut, Copenhagen) was added to half of the leucocyte suspension. To the other half, an equal amount of phosphate buffer 1/15 M was added. The final leucocyte concentration was approximately 9×10^6 cells per ml. The concentration of PPD human in the cultures was 150 μ g per ml. After incubation at 37° C for 30 min, 7 μ l of the leucocyte suspension was placed in the holes of the agarose plates. All the plates were incubated at 37° C in water-saturated atmospheric air. After 24 h, the migration areas were measured by means of a projection microscope. The migration indices were calculated as the ratio between the average of PPD containing cultures and the control cultures.

Lymphocyte Transformation Test (LTT)

Approximately 20 ml of venous blood was drawn into a test tube containing phenol-free heparin in order to produce a final concentration of 20 IU per

ml. The blood was diluted 1:1 with TC medium 199 containing 5 per cent inactivated human A Rh positive serum and heparin 20 IU per ml. An amount of 7-8 ml diluted blood was placed on 3 ml of a mixture of Isopaque-Ficoll. The tubes were then centrifuged at 400 g at room temperature for 30 min; after this the mononuclear cells were harvested from the interphase. The cells were then washed twice in TC 199 with 5 per cent inactivated human serum. The cell suspensions were diluted in TC 199 with 15 per cent pooled normal, inactivated human A Rh positive serum without heparin to a concentration of 10^6 lymphocytes per ml. Samples of 2.0 ml of this suspension were placed in culture tubes measuring 15×150 mm. Purified protein derivative (PPD, RT 32, Statens Serum Institut, Copenhagen), poke weed mitogen (PWM, Grand Island Biological Company) and phytohemagglutinin (PHA, Wellcome MR 68) were added in concentrations of 1 μ g per ml, 10 μ l per ml and 10 μ g per ml, respectively. The pH was adjusted with hepes buffer 0.05 M and culture tubes were then incubated in a water bath at 37° C. Cultures were made in triplicate or, if only a few cells were available, in duplicate. The duration of culture was 5 d for PPD and 3 d for PHA and PWM-stimulated cultures. At 24 h before harvesting the cultures, 0.18 μ Ci thymidine-2- C^{14} (specific activity 50 μ Ci per mM) was added. Harvest was performed on Whatman glass fibre filters (GF/83) and the cells were washed with normal saline. The samples were added to 10 ml dioxane scintillation fluid for scintillation counting and the results were given as disintegration per min (DPM) after correction for quenching and efficiency.

Estimation of ATP-ase Activity in Lymphocytes

Fifty ml samples of venous blood were drawn into plastic syringes and immediately heparinized (8 IU/ml) within the syringe. The blood samples were then passed slowly through a 5 cm long column of washed 0.1 mm glass beads in a 10 ml plastic syringe in order to remove the most sticky population of platelets. This prevents aggregation of platelets during the subsequent isopycnic centrifugation which may cause alterations in their sedimentation characteristics. The blood is then diluted with 3 volumes 0.87 per cent NaCl, also heparinized with 8 IU per ml.

A modification of Böyum's method (1) for separation of lymphocytes was then used: 10 ml mixture of Ficoll 9 per cent and Isopaque 33.9 per cent (v/v 24:10) was layered under 30 ml diluted blood in 50 ml siliconized centrifuge tubes by injection through a long blunt needle. After centrifugation at room temperature for 20 minutes at 650 g, the lymphocyte interphase was harvested with siliconized Pasteur pipettes and transferred to another tube. The lymphocyte suspension was then diluted with 0.87 per cent NaCl 1:5 and spun for 10 min at 650 g. The supernatant was discarded and the cell pellet suspended in 10 per cent sucrose and layered under a discontinuous sucrose gradient of 25 ml 16 per cent sucrose and 4 ml 20 per cent sucrose. After centrifuging for 15 min at 150 g, the lymphocytes were obtained from the bottom of the tube, free from

plateless and only in very rare cases contaminated with some red cells. In such cases, a 5 min exposure to a 1 per cent ammonium oxalate solution was sufficient to lyse the remaining cells completely. The cells were finally suspended in 3 ml 0.25 M sucrose and sonicated for 2×15 sec with a MSE sonicator. Great care was taken to keep the tubes and the cell suspensions cool before and during the sonication. Cell counting was performed in each case using a hemocytometer. The lymphocyte preparations contained 98–100 per cent small and medium-sized lymphocytes, the remainder being polymorphonuclear leucocytes and monocytes. The resulting homogenate was centrifuged for 10 min at 100 g, and aliquots of the supernatant were used for enzyme assay. The protein concentration of the homogenates was determined by Lowry's method (10). The homogenates, containing a heterogenous microsomal fraction of lymphocytes, were incubated for 60 min immediately after preparation using the following assay system: 100 mM NaCl, 15 mM KCl, 6 mM $MgCl_2$, 3 mM Tris-ATP in 30 mM Tris-HCl buffer pH 7.4. The final volume was 2.5 ml. All incubations were carried out at 37° C and the reactivation was terminated by adding perchloric acid to achieve a final concentration of 3 per cent. After 5 min at 0° C, the precipitated protein was removed by centrifuging, and the concentration of inorganic phosphorus in the supernatant determined by the method of Fiske & Subbarow (6). Enzymatic activity was expressed as nano-moles inorganic phosphate liberated from the substrate Tris-ATP per hour per mg protein of the homogenate.

The net results were corrected for endogenous inorganic phosphate of the lymphocytes and the nonenzymatic hydrolysis of Tris-ATP. Double incubations and the double determinations were used throughout the whole procedure.

RESULTS

The response of leucocytes to PPD in the LMT appears from Fig. 1. It is seen that no significant change occurred in migration indices during the administration of levamisole. Fig. 2 and Fig. 3 show the response in the LTT. The response to stimulation with PPD and PWM in 5 persons showed a slight decrease, whereas the response to stimulation with PHA in 8 persons showed variable changes. The control cultures showed a small increase in reactivity. The range of responses was wide and no signs of a large suppression or increase in reactivity could be seen.

The ATP-ase activity of lymphocytes from each person before and during administration of levamisole can be seen in

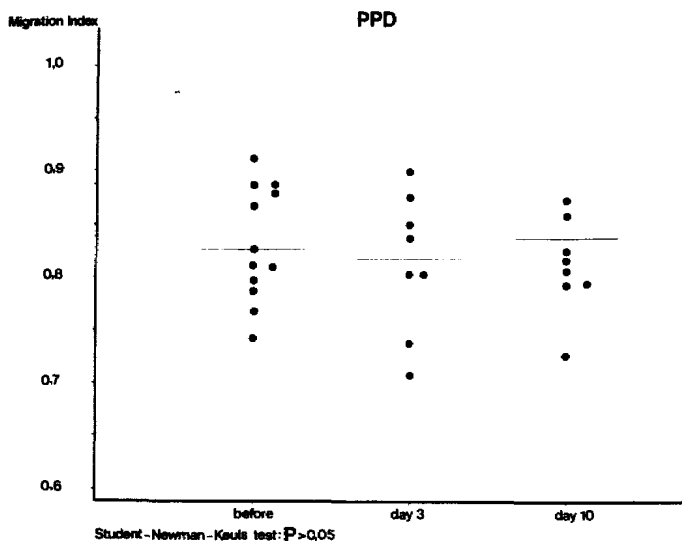


Fig. 1.

Responses of peripheral leucocytes to PPD in agarose migration technique before and after administration of levamisole to healthy volunteers. The results are expressed as migration indices. The average migration index on the various days is indicated by a horizontal line.

Fig. 4. No significant change in enzyme activity could be demonstrated.

DISCUSSION

Studies on the immunopotentiating ability of levamisole have been carried out in mice, either as investigations on macrophages (7, 13), or in experimentally induced tumours (11, 12). There seem to be indications of an increased metabolic and phagocytic activity in the macrophages after administration of levamisole, whereas its effect as a stimulator of immune defense mechanisms against tumours seems doubtful. In humans, levamisole might have an effect in increasing the amount of antibody to vaccination with influenza vaccine (2).

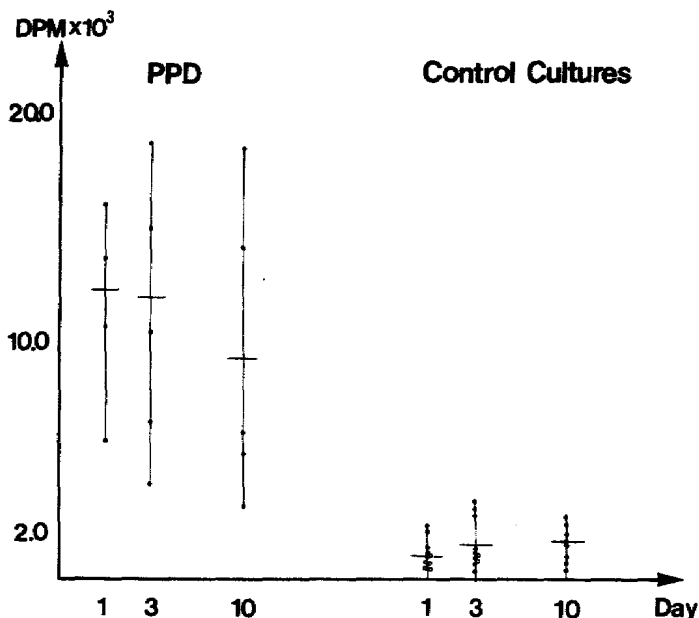


Fig. 2.

Responses of lymphocytes to PPD in lymphocyte transformation tests before and after treatment of the volunteers with levamisole. The results from control cultures are shown to the right. The results are expressed as disintegrations per minute (DPM), after correction for quenching and efficiency.

It has been found to induce a conversion of negative skin tests in cancer patients (15) and also to increase the *in vitro* response of peripheral blood lymphocytes from patients with Hodgkin's disease as measured in a LTT (8).

Our results indicate that levamisole does not change the reactivity in LMT and LTT or the ATP-ase activity of peripheral blood lymphocytes from healthy persons. The results in the LTT are in accordance with previous investigations (2, 9). In one of these (2), a significant increase was seen in the control cultures after levamisole administration;

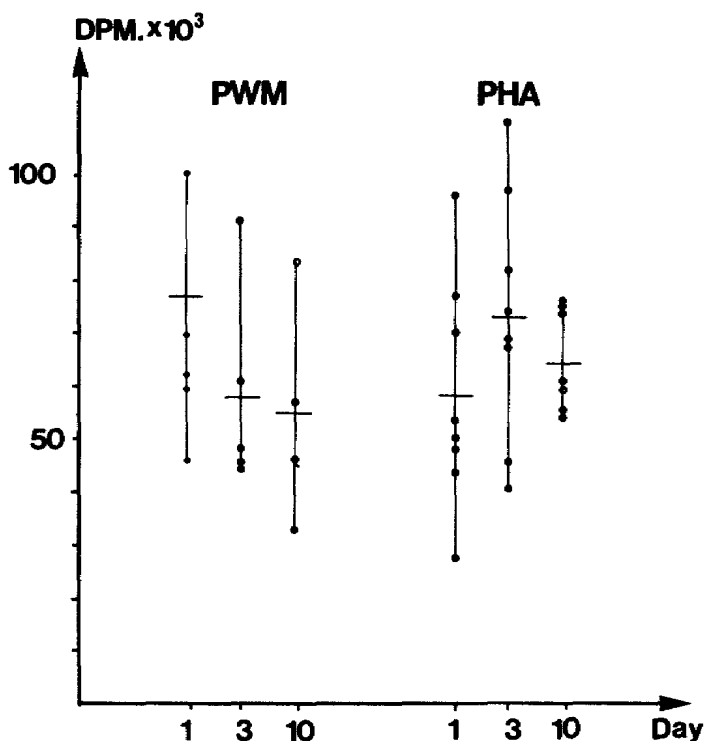


Fig. 3.

Responses of lymphocytes to PWM and PHA in lymphocyte transformation tests before and after treatment of the volunteers with levamisole.

whereas the increase in our control cultures was not significant (paired t-test).

Investigations on LMT and ATP-ase activity during levamisole administration have not been reported. The results from the LMT indicate no increased inhibition as an expression of greater sensitivity of leucocytes to PPD or an increased production migration inhibitor factor (MIF). A general stimulation of lymphocyte ATP-ase activity could not be found in our investigations in normal persons. ATP-ase

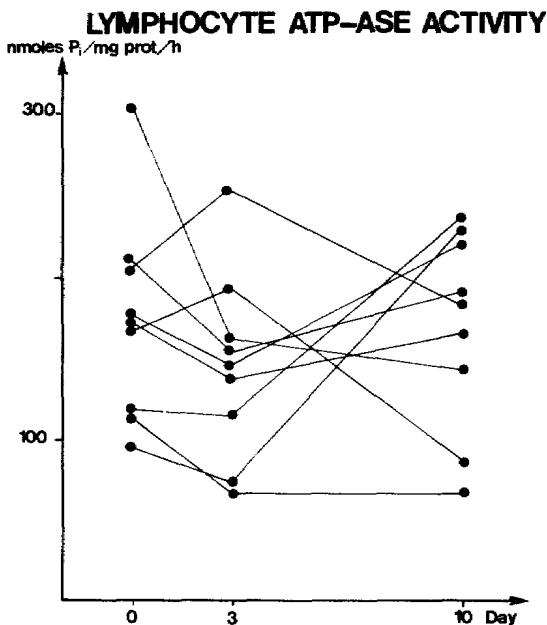


Fig. 4.

ATP-ase activity of lymphocytes before, 3 days after and 10 days after ingestion of levamisole by healthy volunteers.

activity of lymphocytes as assayed in these experiments has earlier been demonstrated to be mitochondrial associated (5). Elevation of activity has been demonstrated in patients with various malignant tumours (5), and it is possible that lymphocytes submitted to tumour-associated antigens in this way could be activated.

It seems, therefore, that levamisole can only influence the reactivity of mononuclear cells when they exhibit a decreased reactivity. Whether the potentiating effect of levamisole on these cells will show a positive effect is, at the moment, not known.

SUMMARY

In vitro parameters of cellular immune reactions were followed in 10 healthy individuals during administration of levamisole. Leucocyte migration tests, lymphocyte transformation tests and ATP-ase activity of peripheral blood lymphocytes were investigated. Levamisole did not influence resting human lymphocytes or the stimulation response of lymphocytes. The metabolic activity of lymphocytes expressed by their ATP-ase activity was not significantly changed after levamisole administration.

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REFERENCES

1. Böyum, A. (1968): Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. clin. Lab. Invest.* 21 (suppl. 97), 77-89.
2. Brugmans, J. et al.: Restoration of host defence mechanisms in man by levamisole. *Life Science* (in press).
3. Carpenter, R. R., Barsales, P. B. & Ganchan, R. P. (1968): Antigen induced inhibition of cell migration in agar gel, plasma clot and liquid media. *J. reticuloendoth. Soc.* 5, 472-483.
4. Clausen, J. (1973): Leucocyte migration technique: some technical details. *Acta allergol.* 28, 351-364.
5. Ellegaard, J., Dimitrov, N. V. (1972): ATP-ase activity of lymphocytes from normal individuals and patients with cancer. *Cancer* 30, 881-884.
6. Fiske, C. H. & Subbarow, Y. (1925): The colorimetric determination of phosphorus. *J. biol. Chem.* 66, 375-400.
7. Hoebeke, J.: Unpublished data.
8. Levo, Y.: Restoration of cellular immune response by levamisole in patients with Hodgkin's disease. Janssen Pharma. Clinical Research Report.
9. Louwagie, A. C. (1972): Tuberculin test, lymphocyte cultures and serum analysis during 4-week treatment with levamisole. Janssen Pharma. Clinical Research Report.

10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951): Protein measurement with folin phenol reagent. *J. biol. Chem.* 193, 265-275.
11. Potter, C. W. et al. (1974): Levamisole inactive in treatment of four animal tumours. *Nature* 249, 567-569.
12. Renoux, G. & Renoux, M. (1972): Levamisole inhibits and cures a solid malignant tumour and its pulmonary metastases in mice. *Nature New Biology* 240, 217-218.
13. Schulze, H. J. (1972): The effect of levamisole on the phagocytising abilities of peritoneal macrophages of the mouse. Lecture held at the 79th meeting of the German Society for Internal Medicine, Wiesbaden.
14. Thienpont, D. et al. (1966): Tetramisole, a new, potent broad spectrum anthelmintic. *Nature* 209, 1084-1086.
15. Tripodi, D., Parks, L. C. & Brugmans, J. (1973): Drug induced restoration of cutaneous delayed hypersensitivity in anergic patients with cancer. *N. Engl. J. Med.* 289, 354-357.
16. Verhaegen, H., De Cree, J. & Verbruggen, J. (1973): Immune responses in elderly cuti-negative subjects and the effects of levamisole. Lecture held at the 79th meeting of the German Society of Internal Medicine, Wiesbaden.

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