

EFFECT OF D-PENICILLAMINE AND LEVAMISOLE ON HUMAN LYMPHOCYTES IN VITRO

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SYNOPSIS

The effects of D-penicillamine and levamisole on the in vitro response of human lymphocytes to two known mitogens, phytohaemagglutinin (PHA) and pokeweed mitogen (PWM), were investigated. The effects observed were both dose-dependent and time-dependent. D-penicillamine (42-84 ug/ml) and levamisole (0.2-20 ug/ml) enhanced the mitogenic response. Both compounds were suppressive at higher doses. These effects were most clearly seen at 96 hours of culture with PHA and at 120 hours with PWM and was also more obvious with PWM as the mitogen. Neither compound had any effect in the absence of mitogen. Lymphocytes from patients with rheumatoid arthritis were also studied. Lymphocytes from patients who did not respond or responded poorly to D-penicillamine therapy showed a similar effect of D-penicillamine as that observed with normal lymphocytes. Patients who responded well to therapy showed a greatly reduced PHA response and little effect of D-penicillamine in vitro. The significance and importance of these findings are discussed.

INTRODUCTION

Both D-penicillamine and levamisole are immuno-modulating drugs which have recently been used successfully in the treatment of various autoimmune diseases such as rheumatoid arthritis (1, 2, 3) and systemic lupus erythematosus (4, 5). Although both these agents have been shown to potentiate immune responses (6, 7) other studies have failed to show any effect of the drugs (8, 9) and their exact mechanism of action remain unknown. Considering the complexity and multivariable aspects of in vivo systems, the use of in vitro methods seems appropriate in studies designed to investigate the effects of immuno-modulating agents on cells of the immune system. The purpose of the present study was to define more accurately the dose-response and time effects of D-penicillamine and levamisole on cell-mediated immunity in vitro as assessed by human lymphocyte blastogenic responses to known mitogens.

MATERIALS AND METHODS

Donors and patients. Blood samples were obtained from normal healthy donors attending the Blood Bank, University Hospital. Blood was also obtained from patients with rheumatoid arthritis which was diagnosed according to standard criteria of the American Rheumatism Association (10).

Blood collection. 4-5 mls of blood was collected into sterile glass tubes containing 0.2 ml of sterile 10% EDTA and agitated immediately to prevent clotting.

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Lymphocyte isolation. Peripheral blood lymphocytes were isolated by the method of Boyum (11). Diluted blood (4 ml blood and 4 ml balanced salt solution, BSS) was gently layered on to 3 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) in siliconised glass tubes. The tubes were then spun at 400 g (MSE bench centrifuge) for 25 minutes at room temperature. After removing the upper layer of plasma the mononuclear cell layer at the interface was harvested with a clean pasteur pipette and washed 3 times with BSS. The lymphocytes were re-suspended in BSS and a sample taken and counted in a Neubauer counter. Viability was determined by trypan blue exclusion.

Lymphocyte culture. Human lymphocytes were cultured in vitro with D-penicillamine and levamisole (both from Sigma Chem. Co., St. Louis, Mo., U.S.A.) in the presence and absence of two mitogens, phytohaemagglutinin (PHA) (Wellcome Laboratories, Beckenham, England) and pokeweed mitogen (PWM) (Grand Island Biological Co., New York, U.S.A.). Cultures were carried out in 96-well, round-bottomed tissue culture plates (Linbro Scientific, Conn, U.S.A.) in RPMI-1640 culture medium (Flow Laboratories, Virginia, U.S.A.) containing 10% foetal calf serum and antibiotics. Lymphocytes were cultured for various periods of time at a concentration of 10^5 cells/well in a 5-10% CO₂ in air atmosphere at 37°C. Approximately 16 hours before harvesting of cultures 1 uCi of ³H-thymidine (Radio-chemical Centre, Amersham) was added to each well. The contents of the culture wells were harvested on to glass fibre discs using an automatic cell harvester (Dynatech Co., U.K.). Discs were dried overnight at 37°C and then counted in a liquid scintillation counter (Packard Tri Carb, U.S.A.).

Analysis of data. ³H-thymidine incorporation was expressed as mean counts per minute (CPM) for triplicate cultures. Standard errors of the mean were also determined.

RESULTS

The effects of D-penicillamine on normal human lymphocytes stimulated with phytohaemagglutinin (PHA) is depicted on Fig. 1. A dose and time-dependent effect was observed with stimulation of the lymphocyte response at 84 ug/ml and suppression with doses of 210-240 ug/ml (Fig. 1). These effects were more obvious after 96 hrs of culture (Fig. 1). With pokeweed mitogen (PWM), significant enhancement of response was noted with doses of 8.4-84 ug/ml whereas suppression was seen at concentrations of 420-840 ug/ml (Fig. 2). These effects were only seen at 120 hours of culture prior to which the drug had little effect or was even suppressive (at 96 hours) (Fig. 2). Effect of levamisole on PHA-stimulated lymphocytes is shown on Fig. 2. Stimulation of response was noted at 72 hrs with doses of 0.2 to 20 ug/ml while doses of 200 and 2000 ug/ml were strongly suppressive especially at 96 hours (Fig. 3). With PWM, significant stimulation was observed with doses between 0.2 - 200 ug/ml levamisole while suppression was noted with a dose of 2000 ug/ml (Fig. 4). As with D-penicillamine (see Fig. 2), these effects of levamisole were best seen at 120 hours of culture (Fig. 4). The effect of D-penicillamine on the PHA response of lymphocytes from 6 patients with rheumatoid arthritis was also studied. It was noted that lymphocytes from two patients who did not respond or responded only moderately to D-penicillamine therapy showed a similar effect of D-penicillamine as normal lymphocytes (Fig. 5) ie. stimulation with a dose of 84 ug/ml and suppression at higher doses of 420 ug/ml. Interestingly, it was also observed that lymphocytes from two other patients who were good responders to D-penicillamine therapy showed a greatly reduced PHA response and only a slight effect of D-penicillamine at the various doses tested (Fig. 5). It should also be pointed out that neither D-penicillamine nor levamisole alone (at various doses) had any effect on

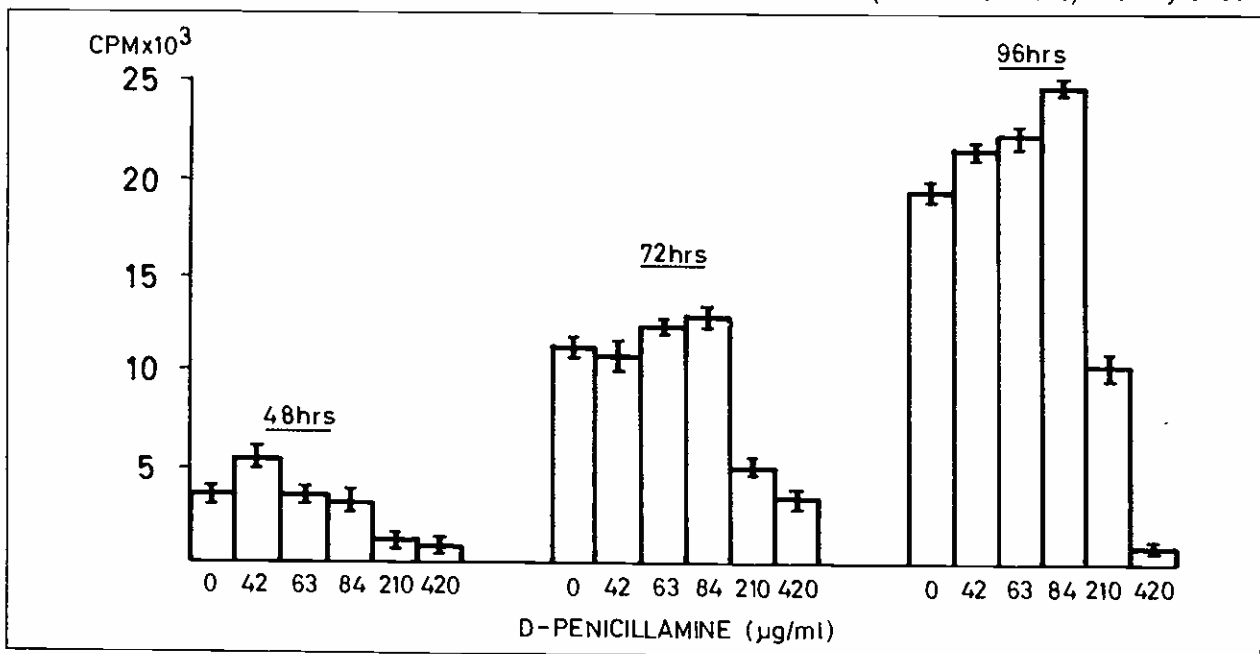


Figure 1. Effect of D-penicillamine on human lymphocytes stimulated with phytohaemagglutinin (PHA). Lymphocytes were cultured with various concentrations of D-penicillamine and PHA at a final dilution of 1 in 250 (36 ug/ml) and harvested at various times after commencement of culture.

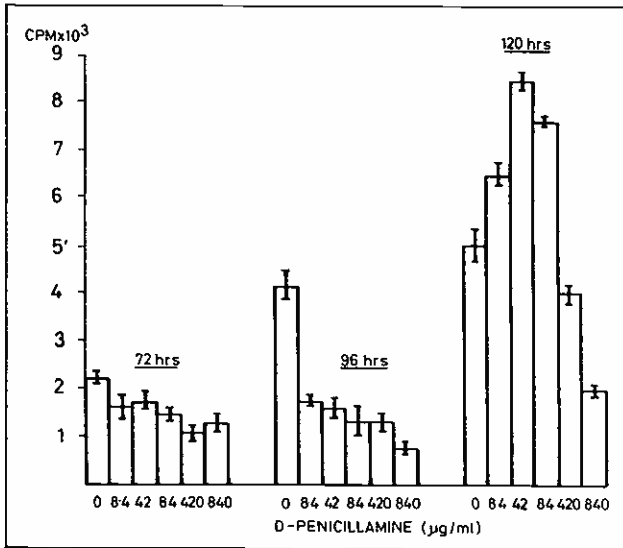


Figure 2. Effect of D-penicillamine on human lymphocytes stimulated with pokeweed mitogen (PWM). Lymphocytes were cultured with various concentrations of D-penicillamine and PWM at a final dilution of 1 in 10 (20 ug/ml) and harvested at various times after commencement of culture.

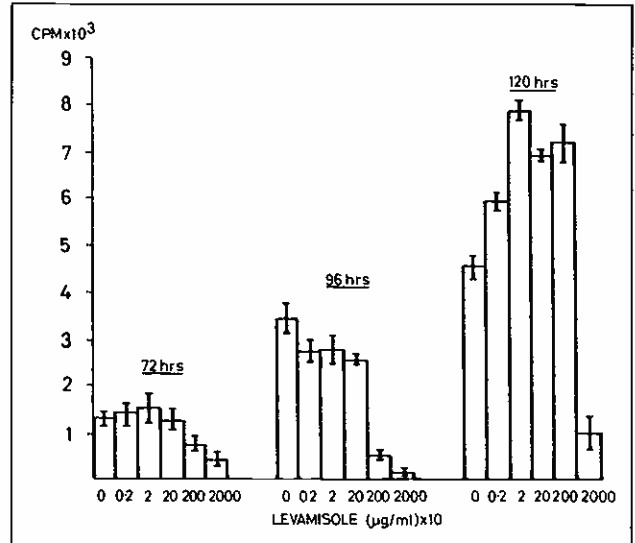


Figure 4. Effect of levamisole on human lymphocytes stimulated with pokeweed mitogen (PWM). Lymphocytes were cultured with various concentrations of levamisole and PWM at a final dilution of 1 in 10 (20 ug/ml) and harvested at various times after commencement of culture.

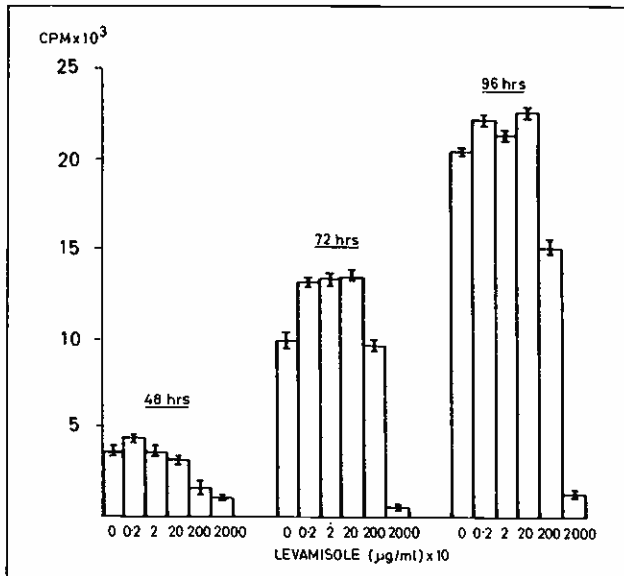


Figure 3. Effect of levamisole on human lymphocytes stimulated with phytohaemagglutinin (PHA). Lymphocytes were cultured with various concentrations of levamisole and PHA at a final dilution of 1 in 250 (36 ug/ml) and harvested at various times after commencement of culture.

human lymphocytes in the absence of PHA or PWM (results not shown).

DISCUSSION

Results presented in this study clearly show that both D-penicillamine and levamisole affected human lymphocyte responses to mitogens in a manner which was both dose-dependent and time-dependent. With D-penicillamine, enhancement of lymphocyte responses was observed with doses between 42-84 ug/ml and suppression at higher doses of > 210 ug/ml. Levamisole also enhanced the response in the dose range 0.2-20 ug/ml and was suppressive at doses of > 200 ug/ml. The enhancing effect of both drugs seem to be more obvious with PWM as the mitogen rather

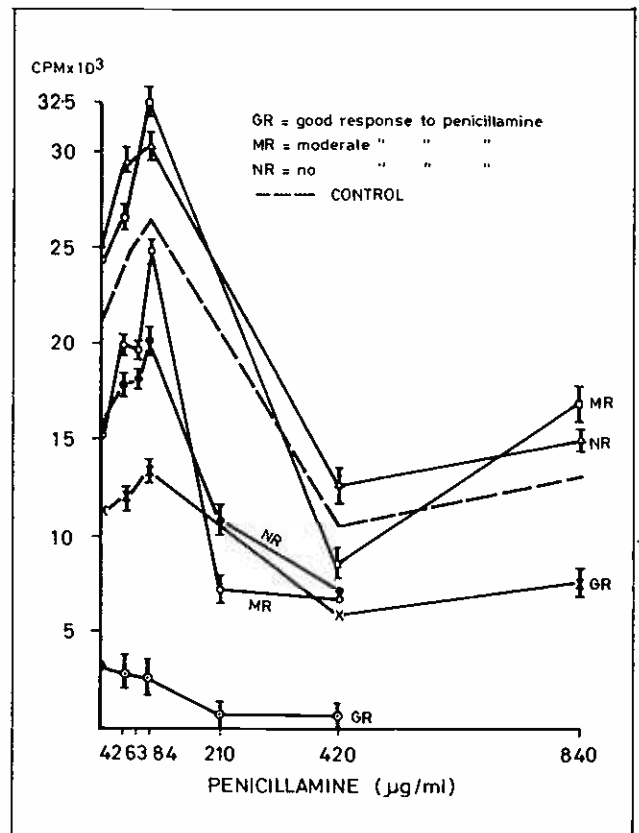


Figure 5. Effect of D-penicillamine on human lymphocytes from patients with rheumatoid arthritis stimulated with phytohaemagglutinin (PHA). Lymphocytes were cultured with various concentrations of D-penicillamine and PHA at a final dilution of 1 in 250 (36 ug/ml). Cultures were harvested after 96 hours.

than PHA. The effects noted with both drugs were also most clearly seen at 120 hours of culture with PWM and at 96 hours with PHA. At 96 hours of culture with PWM, for example all doses tested were suppressive. As mentioned before, neither drug alone had any stimulatory activity.

The results presented are in general agreement with those obtained by other investigators. Maini and Roffe (12) showed enhancement of lymphocyte responses *in vitro* at lower concentrations of D-penicillamine and Roath and Wills (13) reported suppression with doses of 200-1000 ug/ml. With levamisole, augmentation of lymphocyte responses was observed at various concentrations and suppression once again noted with the higher doses (14-16). Many of these earlier studies, however, looked at the effects of D-penicillamine and levamisole at one time point only. As pointed out previously (17) failure to take account of the time-course of the response can lead to erroneous conclusions regarding the properties of these compounds, as expressed towards stimulated lymphocytes. For example, D-penicillamine has been attributed with an entirely immunosuppressive role (13). Our finding that augmentation of the response was more marked with PWM was also noted by Sampson and Lui (18). This may be a reflection of the fact that PWM stimulates both T and B lymphocytes whereas PHA is mainly a T cell mitogen. However, a more likely explanation for this observation is the fact that the overall magnitude of the response to PHA is much stronger than that to PWM. Because of this high 'background' of PHA stimulation, any enhancement of the response would be less apparent when compared to PWM stimulation where the base-level response to the mitogen alone is approximately only one-third of that observed with PHA. Also in agreement with previous findings (14-17) was our observation that both agents had no effect *in vitro* in the absence of mitogen i.e. they were non-mitogenic by themselves.

In relation to the PHA response of lymphocytes from patients with rheumatoid arthritis it was noted that the response was depressed, marginally depressed or slightly elevated (see Fig. 5). Consistent with this finding is the suggestion that in these patients both hypo- and hyper-reactive lymphocyte populations are present (19). In relation to the effects of D-penicillamine we noted that patients who responded well to D-penicillamine therapy showed markedly depressed PHA responses. It is probable that this is due to the fact that the lymphocytes in these patients have already been pre-stimulated by the drug *in vivo* and consequently fail to give a good response *in vitro* to a potent mitogen such as PHA. Moreover, their pre-exposure to the drug *in vivo* makes them less sensitive to penicillamine stimulation. Conversely, lymphocytes from poor or moderate responders to D-penicillamine therapy are presumably non-stimulated and can thus respond to PHA *in vitro*. In contrast to the poor *in vivo* response, lymphocytes from two of these patients showed enhancement by D-penicillamine *in vitro* after PHA stimulation. The reason for this is not clear but may be related to differences in metabolism of the drug under *in vivo* and *in vitro* conditions.

The exact mode of action of D-penicillamine and levamisole remains largely unknown. In relation to the present *in vitro* study, it has been suggested that D-penicillamine may affect the lymphocyte membrane, resulting in increased receptor affinity for

the mitogen and enhanced response (6). Other possibilities, however, should be kept in mind e.g. stimulation of macrophage function, direct metabolic effects as a result of drug entry into the cell and non-specific 'conditioning' of the culture media thus facilitating a more optimal response to mitogens. On the other hand, the suppressive effect at higher doses of D-penicillamine may be due to non-specific interference with binding of mitogen (6). It is not due to toxic effects as a check on lymphocyte viability at these high doses revealed a viability of 95%. The *in vitro* effect of levamisole on lymphocytes is thought to involve a depression of intracellular cAMP levels which in turn causes a lowered threshold of responsiveness to stimuli (7, 20). In relation to its suppressive effects at the higher doses, cytotoxicity was observed in the present study at levamisole concentrations of > 200 ug/ml.

It is also important to point out that the exact significance of these *in vitro* results in relation to the *in vivo* effects of the drugs is not clear. We may, however, infer from the pharmacokinetic properties of D-penicillamine (21) that it is the stimulatory concentrations which are attained under *in vivo* conditions. Clearly many questions remain unanswered and other areas of investigations need to be pursued e.g. effect of these compounds on the phagocyte system and on functional T cell subsets (e.g. helper, suppressor), the role of lymphokines and the presence of specific drug receptors on the lymphocyte membrane. These investigations are currently being pursued in our laboratory.

REFERENCES

1. Multicentre Trial Group: Controlled trial of D-penicillamine in severe rheumatoid arthritis. *Lancet* 1973; 1: 275-80.
2. Multicentre Trial Group: Efficacy of levamisole in rheumatoid arthritis. *Lancet* 1978; 1007-12.
3. Dixon A St J, Davies J, Dormandy TL et al. Synthetic D (-) Penicillamine in rheumatoid arthritis. *Ann Rheum Dis* 1975; 34: 416-21.
4. Gordon BL and Yanagihara R: Treatment of systemic lupus erythematosus with the T-cell immunopotentiator levamisole: A follow-up report of sixteen cases under treatment for a minimum period of 4 months. *Proc XII Singapore-Malaysia Congress of Medicine* 1977; 12: 517-29.
5. Feng PH, Oon CJ, Yo SL, Lau YK: Levamisole in the treatment of systemic lupus erythematosus - preliminary results. *Singapore Med J* 1978; 19: 120-4.
6. Arrigoni-Martelli E, Bramm E, Binderup L: Immunostimulant activity of D-penicillamine. *Drugs Exptl Res* 1977; 2: 189-95.
7. De Cree J and Symoens J: Immunological profile of levamisole and its clinical application in man. In: JL Turk and D Parker, eds. *Drugs and Immune Responsiveness*. London: MacMillan Press, 1979; 85-117.
8. Liyanage SP and Currey HLF: Failure of D-penicillamine to modify adjuvant arthritis or immune response in rat. *Ann Rheum Dis* 1972; 31: 521.
9. Copeland D, Stewart T, Harris JE: Effect of levamisole on *in vitro* human lymphocyte transformation. *Cancer Chemother Rept* 1974; 58: 167-70.
10. Ropes MW, Bennett GA, Cobb S, Jacox R, Jessar RA: Revision of diagnostic criteria for rheumatoid arthritis. *Ann Rheum Dis* 1959; 18: 49-55.
11. Boyum A: Separation of leucocytes from blood and bone

- marrow. *Scand J Clin Lab Invest* 1968; 21 (Suppl 97): 91-106.
12. Maini RN and Roffe L: D-penicillamine and lymphocyte function in rheumatoid arthritis: the inhibitory and augmenting effects of D-penicillamine in vitro on phytohaemagglutinin-induced lymphocyte transformation. In: E Munthe ed. *Penicillamine Research in Rheumatoid Disease*. Oslo: Fabritius & Sonner, 1976; 172-84.
 13. Roath S and Wills R: The effects of penicillamine on lymphocytes in culture. *Postgrad Med J* 1974; 50 (Suppl 2): 56-9.
 14. Woods WA, Siegel MJ, Chirigos MA: In vitro stimulation of spleen cell cultures by poly I: poly C and levamisole. *Cell Immunol* 1974; 14: 327-31.
 15. Hadden JW, Coffey RG, Hadden EM: Effects of levamisole and imidazole on lymphocyte proliferation and cyclic nucleotide levels. *Cell Immunol* 1975; 20: 98-103.
 16. Lichtenfeld JL, Desner MR, Wiernik PH, Mardiney MR: Modulating effects of levamisole on human lymphocyte response in vitro. *Cancer Treatment Rep* 1976; 60: 571-4.
 17. Kendall PA and Hutchins D: The effect of thiol compounds on lymphocytes stimulated in culture. *Immunology* 1978; 35: 189-201.
 18. Sampson D and Lui A: The effect of levamisole on cell-mediated immunity and suppressor cell function. *Cancer Res* 1976; 36: 952-5.
 19. Maini RN, Glass DN, Scott JT: *Immunology of the Rheumatic Diseases*. London: Edward Arnold, 1977.
 20. Thompson RA: *The Practice of Clinical Immunology*. London: Edward Arnold, 1978.
 21. Ruiz-Torres A: Zur pharmkokinetik und zum staffwechsel von D-und L-penicillamin. II Mitteilung: Verteilung von D-und L-penicillamin – ¹⁴C in organismus der ratte nach peroraler verabfolgung. *Arzneimittel Forsch* 1974; 24: 1043-6.