Br. J. Pharmac. (1978), **63**, 119–123.

THE EFFECTS OF D-PENICILLAMINE AND LEVAMISOLE ON LEUCOCYTE CHEMOTAXIS IN THE RAT

F.M. CUNNINGHAM, A.W. FORD-HUTCHINSON, A.M. OLIVER, M.J.H. SMITH & J.R. WALKER

Biochemical Pharmacology Research Unit, Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London, SE5 8RX

1 The administration of D-penicillamine (25 mg/kg) or levamisole (5 mg/kg) had no effect on leucocyte emigration into the exudates formed in inert sponges implanted in normal rats.

2 In rats, previously sensitized to *Bordetella pertussis* and implanted with sponges containing *pertussis* vaccine, an increased leucocyte migration into the exudates occurred; this was significantly enhanced by the administration of the drugs.

3 Neither drug *in vitro* affected the chemotaxis of rat polymorphonuclear leucocytes although random migration was significantly increased by levamisole (2 μg to 1 mg/ml). Neither drug affected the chemotaxis of rat mononuclear cells although levamisole (25 μg/ml) significantly increased that of human monocytes.

4 It is concluded that both drugs produce similar effects in an animal model of delayed hypersensitivity and that their clinical antirheumatic actions may have common elements.

Introduction

D-Penicillamine and levamisole are antirheumatic drugs with a late onset of action since several months of treatment may be required to produce an effective clinical response. They also differ from conventional non-steroidal antirheumatic agents in showing no anti-inflammatory effects in animal models (Huskisson, Dieppe, Scott, Trapnell, Balme & Willoughby, 1975). In contrast, their administration in the rat exacerbates the secondary lesions of adjuvant arthritis and increases the response to a delayed hypersensitivity caused by *Bordetella pertussis* vaccine (Arrigoni-Martelli & Bramm, 1975; Dieppe, Willoughby, Huskisson & Arrigoni-Martelli, 1976). Levamisole has been reported to stimulate both humoral and cell-mediated immune responses in man and animals (Renoux & Renoux, 1972; Tripodi, Parkes & Brugmans, 1973). The drug stimulates several functions of human monocytes *in vitro* including chemotaxis (Pike & Snyderman, 1976; Schmidt & Douglas, 1976) and *in vivo* restores the diminished chemotactic responsiveness which occurs in certain human diseases (Wright, Kirkpatrick & Gallin, 1977). Little information is available about the effects of D-penicillamine on similar systems except that it has been reported to inhibit leucocyte chemotaxis *in vitro* (Chwalinska-Sadowska & Baum, 1976). In the present work we have examined the effects of the drugs *in vivo* on the emigration of leucocytes into exudates formed both in inert sponges implanted in normal rats and in sponges containing *Bordetella pertussis* implanted in sensitized rats and their effects *in vitro* on the directed migration of rat polymorphonuclear and mononuclear leucocytes.

A preliminary account of some of this work has been published (Cunningham, Ford-Hutchinson, Smith & Walker, 1978).

Methods

Female albino Wistar rats (150–200 g) were obtained from Oxfordshire Laboratory Animal Colonies, 1976, Ltd. Levamisole ((−)-tetramisole) was a gift from Dr P. Janssen and D-penicillamine was obtained as the free base from the Sigma Chemical Co. *Bordetella pertussis* vaccine was obtained from the Lister Institute and was washed three times in 0.9% w/v NaCl solution (saline) to remove preservatives before use.

Sponge implantation in the rat

The sponge implanation technique used was that described previously (Ford-Hutchinson, Smith, Elliott, Bolam, Walker, Lobo, Badcock, Colledge & Billimoria, 1975) except that each rat received four

sponges soaked either in saline or in a suspension of heat-killed *Bordetella pertussis* (4×10^{10} organisms/ml) in saline. Sponges were removed after 48 h, the cells were removed from the sponge with trypsin and total and differential cell counts performed. Trypsin treatment removes all the cells from the sponges at this time interval (Doherty, Antilla & Dean, 1977).

Delayed hypersensitivity reaction in the rat

Rats were sensitized with *pertussis* vaccine and Freund's incomplete adjuvant (Difco) according to the directions of Dieppe *et al.* (1976). The rats were challenged 12 days later by implantation of sponges containing *pertussis* vaccine. D-Penicillamine (25 and 5 mg/kg) and levamisole (5 and 1 mg/kg) were administered orally, once daily, either 48 h, 24 h and 1 h prior before sensitization or 48 h, 24 h and 1 h before and 24 h after sponge implantation.

Polymorphonuclear leucocyte chemotaxis under agarose

The effects of D-penicillamine and levamisole on polymorphonuclear leucocyte chemotaxis and random migration were assessed using migration under agarose as described by Nelson, Quie & Simmons (1975). Blood was withdrawn from rats by cardiac puncture into a heparinized syringe (20 iu heparin/ml). Dextran (6% w/v clinical grade, average mol. wt. 234,000) was added to a final concentration of 1% w/v and the red cells were allowed to sediment at 20°C for 45 minutes. The leucocytes were collected by centrifugation at 200 g for 20 minutes. Any remaining red cells were removed by hypotonic lysis (0.2% w/v NaCl for 30 s) and the leucocytes were then washed once with saline. The cells were resuspended in Eagle's MEM (minimum essential medium) containing 30 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer, pH 7.4, at a concentration of 4×10^6 white cells/ml and were incubated with concentrations of D-penicillamine and levamisole ranging from 200 ng/ml to 1 mg/ml for 1 h at 37°C. The cells were then centrifuged at 200 *g* for 10 min and resuspended in medium. The cell suspension (5 μl) containing 4.5×10^5 leucocytes was applied to wells punched in agarose plates made up as described by Nelson *et al.* (1975) except that agarose, 1% w/v (Miles Laboratories) was made up in Eagle's MEM buffered to pH 7.4 with 30 mM HEPES buffer and supplemented with 10% heat inactivated rat serum. Heat inactivated (56°C for 30 min) rat serum was used as a control and rat serum activated at 37°C for 1 h with 0.5 mg/ml endotoxin (*E. coli* lipopolysaccharide, 0111:B4, Difco) followed by heat inactivation was used as a chemotactic agent. The plates were incubated at 37°C for 2 h in a humid atmosphere. The cells were fixed and stained as described by Nelson *et al.* (1975) and the distances moved by the cells were measured with the grid on a Projectina Projecting microscope (1 grid unit = 350 μm). The chemotactic index (the distance moved towards the chemotactic

Table 1 The effects of administration of D-penicillamine and levamisole on leucocyte migration into sponges implanted for 48 h in the rat

A Sensitized rats B. pertussis *sponges*	*Dosage (mg/kg)*	*No. of rats per group*	*Total leucocyte count* ($\times 10^2/\mu l$)
Control		11	295 ± 29
Penicillamine[1]	25	10	366 ± 27 $P < 0.05$
Levamisole[1]	5	10	340 ± 34
Penicillamine[2]	25	10	367 ± 36 $P < 0.05$
Levamisole[2]	5	10	370 ± 37 $P < 0.05$
B Non-sensitized rats Sterile sponges			
Control		15	132 ± 13
Penicillamine[2]	25	15	113 ± 16
Levamisole[2]	5	15	122 ± 17
C Non-sensitized rats *B. pertussis* sponges			
Control		5	122 ± 16

Results are expressed as means ± s.e. mean and compared with the control group by *t* test.
[1]Drug administered 48 h, 24 h and 1 h before sensitization.
[2]Drug administered 48 h, 24 h and 1 h before and 24 h after sponge implantation.

agent divided by the distance moved towards the control well) and the chemotactic differential (the distance moved towards the chemotactic agent minus the distance moved towards the control well) were calculated.

Monocyte chemotaxis

The effects of D-penicillamine and levamisole on monocyte chemotaxis were assessed by the modified Boyden chamber technique as described by Pike & Snyderman (1976). Blood was withdrawn by cardiac puncture from rats and monocytes were separated on Hypaque/Ficoll gradients. The cells were incubated for 30 min in Eagle's MEM buffered to pH 7.4 with 30 mM HEPES buffer with drug concentrations ranging from 250 ng to 250 μg/ml.

Results

Chemotaxis in vivo

The addition of *Bordetella pertussis* vaccine to sponges implanted for 48 h in non-sensitized rats produced no change in leucocyte migration (Table 1). Sensitization of the rats, followed 12 days later by challenge with sponges impregnated with *Bordetella pertussis* organisms, produced a 141% increase in white cell migration ($P < 0.001$) and the exudate was predominantly composed of mononuclear cells (67%).

In sensitized rats the administration of D-penicillamine (25 mg/kg) either before sensitization or challenge significantly enhanced leucocyte infiltration into the sponge exudate at 48 h (Table 1). With levamisole (5 mg/kg) this effect was only significant when the drug was administered at the time of challenge. No significant changes in differential cell counts were observed between any of the groups. Administration of D-penicillamine (5 mg/kg) and levamisole (1 mg/kg) using the same dosage schedule produced a smaller nonsignificant enhancement of leucocyte migration. In non-sensitized rats implanted with untreated sponges the same dosage schedule of D-penicillamine and levamisole did not affect the cellular response (Table 1).

Polymorphonuclear leucocyte chemotaxis in vitro

The results from a typical experiment showing the effects of preincubation of rat polymorphonuclear leucocytes with levamisole upon chemotaxis and random migration are shown in Table 2. An apparent effect on chemotaxis can be seen at all doses studied. The effect, however, was due to an increase in random migration and no significant changes in the chemotactic differentials were seen. The result was reproducible in subsequent experiments. Similar experiments were performed with D-penicillamine with concentrations ranging from 20 ng/ml to 1 ng/ml. No significant changes in the directed or random migration of cells exposed to this drug were observed.

Monocyte chemotaxis in vitro

In contrast to the stimulatory effect of levamisole on human monocyte chemotaxis observed by other workers (Pike & Snyderman, 1976; Schmidt & Douglas, 1976) and confirmed in the present work, no significant effect of either levamisole or D-penicillamine was observed on rat monocyte chemotaxis (Table 3).

Table 2 The effects of preincubation with levamisole upon the directed and random migration of rat polymorphonuclear leucocytes

Levamisole concentration	*Directed migration*	*Random migration*	*Chemotactic index*	*Chemotactic differential*
Control	1.28 ± 0.08	0.32 ± 0.05	4.37 ± 0.26	0.98 ± 0.05
20 ng/ml	1.88 ± 0.19**	0.54 ± 0.07*	4.43 ± 1.03	1.37 ± 0.26
2 μg/ml	1.78 ± 0.08**	0.68 ± 0.07**	2.72 ± 0.24**	1.10 ± 0.09
20 μg/ml	1.66 ± 0.07	0.56 ± 0.01**	3.05 ± 0.18**	0.94 ± 0.20
100 μg/ml	1.78 ± 0.15**	0.70 ± 0.12**	2.84 ± 0.33**	1.08 ± 0.09
1 mg/ml	1.92 ± 0.07**	0.80 ± 0.07**	2.46 ± 0.18**	1.12 ± 0.09

The results are expressed as means ± s.e. mean with 12 determinations for the control value and 6 for each of the drug concentrations and were compared by the *t* test. The results for directed migration, random migration and chemotactic differential are expressed as divisions on the Projectina microscope grid (1 grid unit = 350 μm).
*$P < 0.05$; **$P < 0.01$.

Discussion

Neither D-penicillamine nor levamisole affected the accumulation of leucocytes into the exudates formed in untreated sponges implanted subdermally in the rat when the drugs were administered at time intervals ranging from 48 h before to 24 h after implantation. Other non-steroidal antirheumatic agents, such as aspirin and indomethacin, produce significant inhibitions of leucocyte emigration in this model (Walker, Smith & Ford-Hutchinson, 1976). This finding agrees with the results of other workers (Huskisson *et al.*, 1975) that D-penicillamine and levamisole do not exhibit anti-inflammatory actions in conventional animal models. When the rats were sensitized by an initial injection of *pertussis* vaccine and implanted subsequently with sponges containing the vaccine there was an increased accumulation of leucocytes in the sponge exudates at 48 hours. The leucocyte emigration was further enhanced by the administration of D-penicillamine either at the time of sensitization or at sponge implantation and by the administration of levamisole during the latter period. Similar results have been reported in a related animal model in which the emigration of leucocytes into the pleural space was measured (Dieppe *et al.*, 1976).

The mechanism of this effect of both drugs has not been established. One possibility is that their administration directly stimulates the chemotaxis of polymorphonuclear and mononuclear leucocytes into the sponge exudates. Despite the finding that levamisole stimulates human monocyte chemotaxis *in vitro* (Table 3; Pike & Snyderman, 1976; Schmidt & Douglas, 1976) neither drug produced significant effects on rat leucocyte chemotaxis *in vitro* or into untreated sponges implanted subdermally in the rat *in vivo*. This therefore cannot be the explanation for the increased leucocyte accumulation observed in exudates from drug-treated sensitized rats. The target for the drugs could either be the lymphocytes responsible for the expression of the delayed hypersensitivity response or bone marrow derived monocytes which act as accessory cells in reactions of this type (Turk, 1975). In this context both drugs have been shown *in vitro* to stimulate rat lymph node cells to mitosis and to potentiate their response to mitogen (concanavalin A). Levamisole has been reported to stimulate lymphocytes directly whereas the involvement of macrophages is essential for the stimulatory action of D-penicillamine (Arrigoni-Martelli, Binderup & Bramm, 1977).

A second query is the possible relation of the effects of the drugs in the *pertussis* sponge model to their other actions. There is considerable evidence that administration of levamisole stimulates both humoral and cell-mediated immune responses but there is little evidence that either D-penicillamine shares these properties or that these effects relate to the antirheumatic actions of the drug. However, the present work shows that both levamisole and D-penicillamine can produce similar effects in an animal model of delayed hypersensitivity suggesting that their overall antirheumatic action may have common elements.

Our thanks are due to Dr P. Janssen, Janssen Pharmaceutica, 2340 Beerse, Belgium for a gift of Levamisole.

Table 3 The effects of preincubation with levamisole and D-penicillamine upon rat and human monocyte chemotaxis

Drug concentration	*D-penicillamine rat monocytes*	*Levamisole rat monocytes*	*Levamisole human monocytes*
Medium alone	21.6 ± 1.2	22.4 ± 1.5	44.7 ± 2.6
250 ng/ml	19.5 ± 1.4	26.9 ± 1.3	—
2.5 μg/ml	19.3 ± 1.1	17.7 ± 1.8	51.9 ± 2.7
25 μg/ml	19.9 ± 1.9	18.9 ± 1.0	60.9 ± 2.9*
250 μg/ml	24.3 ± 3.1	26.9 ± 1.2	—
Random migration (no chemotactic factor)	2.1 ± 0.5*	2.7 ± 0.5*	20.8 ± 1.6*

Results are expressed as the average number of monocytes in 10 experiments migrating completely through a 5.0 μm polycarbonate filter per oil immersion field (×1200) ± s.e. mean.
* $P < 0.001$ compared with medium alone.

References

ARRIGONI-MARTELLI, E., BINDERUP, L. & BRAMM, E. (1977). Role of macrophages in D-penicillamine-induced stimulation of DNA synthesis in lymph node cells *in vitro*. In *Perspectives in Inflammation*. ed. Willoughby, D.A., Giroud, J.P. & Velo, G.P. pp. 295–301. London: MTP Press.

ARRIGONI-MARTELLI, E. & BRAMM, E. (1975). Investigations in the influence of cyclophosphamide, gold, sodium thiomalate and D-penicillamine on nystatin oedema and adjuvant arthritis. *Agents and Actions*, **5**, 264–267.

CHWALINSKA-SADOWSKA, H. & BAUM, J. (1976). The effect of D-penicillamine on polymorphonuclear leucocyte function. *J. clin. Invest.*, **58**, 871–879.

CUNNINGHAM, F.M., FORD-HUTCHINSON, A.W., SMITH, M.J.H. & WALKER J.R. (1978). Effects of D-penicillamine and levamisole on delayed hypersensitivity reactions in the rat and mouse. *Br. J. Pharmac.*, **62** (in press).

DIEPPE, P.A., WILLOUGHBY, D.A., HUSKISSON, E.C. & ARRIGONI-MARTELLI, E. (1976). Pertussis vaccine pleurisy: a model of delayed hypersensitivity. *Agents and Actions*, **6**, 618–621.

DOHERTY, N.S., ANTILLA, M. & DEAN, P.B. (1977). Penetration of naproxen and salicylate into inflammatory exudates in the rat. *Ann. Rheum. Dis.*, **36**, 244–248.

FORD-HUTCHINSON, A.W., SMITH, M.J.H., ELLIOTT, P.N.C., BOLAM, J.P., WALKER, J.R., LOBO, A.A., BADCOCK, J.K., COLLEDGE, A.J. & BILLIMORIA, F.J. (1975). Effects of a human plasma fraction on leucocyte emigration into inflammatory exudates. *J. Pharm. Pharmac.*, **27**, 106–112.

HUSKISSON, E.C., DIEPPE, P.A., SCOTT, J., TRAPNELL, J., BALME, M.W. & WILLOUGHBY, D.A. (1976). Immunostimulant therapy with levamisole for rheumatoid arthritis. *Lancet*, **i**, 393–395.

NELSON, R.D., QUIE, P. G. & SIMMONS, R. L. (1975). Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leucocytes and monocytes. *J. Immunol.*, **415**, 1650–1656.

PIKE, M.C. & SNYDERMAN, R. (1976). Augmentation of human monocyte chemotactic response by levamisole. *Nature*, **261**, 136–137.

RENOUX, G. & RENOUX, M. (1972). Levamisole inhibits and cures a solid malignant tumour and its pulmonary metastases in mice. *Nature, New Biol.*, **240**, 217–218.

SCHMIDT, M.E. & DOUGLAS, S.D. (1976). Effects of levamisole on human monocyte function and immunoprotein receptors. *Clin. Immunol. Immunopath.*, **6**, 299–305.

TRIPODI, D., PARKES, L.C. & BRUGMANS, J. (1973). Drug-induced restoration of cutaneous delayed hypersensitivity in anergic patients with cancer. *New Engl. J. Med.*, **289**, 344–347.

TURK, J.L. (1975). Delayed hypersensitivity. *Frontiers of Biology*, vol. 4. Amsterdam: Elsevier, North Holland.

WALKER, J.R., SMITH, M.J.H. & FORD-HUTCHINSON, A.W. (1976). Anti-inflammatory drugs, prostaglandins and leucocyte migration. *Agents and Actions*, **6**, 602–606.

WRIGHT, D.G., KIRKPATRICK, C.H. & GALLIN, J.I. (1977). Effects of levamisole on normal and abnormal leucocyte locomotion. *J. clin. Invest.*, **59**, 941–950.

(Received November 4, 1977. Revised January 10, 1978.)