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THE EFFECTS OF SOME ANTIRHEUMATIC DRUGS ON AN *in vitro* MODEL OF HUMAN POLYMORPHONUCLEAR LEUCOCYTE CHEMOKINESIS

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1 A rapid, reproducible *in vitro* assay for studying the chemokinetic movement of human polymorphonuclear leucocytes (PMNs) is described. Two synthetic peptides, formyl methionyl-leucyl-phenylalanine (FMLP) and formyl methionyl-phenylalanine (FMP), were used as standard chemokinesins.

2 Maximal chemokinetic movement was observed with peptide concentrations of 2.5 nM (FMLP) and 100 μM (FMP). EC_{50} values of 650.0 ± 60.0 pM and 27.0 ± 3.5 μM respectively are similar to those reported for chemotactic activity of the peptides in micropore filter assays.

3 The PMN chemokinetic response to FMLP was enhanced by histamine (100 nM) and vitamin C (2.5 μM).

4 Human serum albumin was shown to induce chemokinesis but to antagonize the response to FMLP in a dose-related fashion. Fibrinogen similarly antagonized the cell response to peptide.

5 Levamisole (250 nM to 2.5 μM) significantly potentiated the chemokinetic responses to FMLP and FMP in a dose-related manner. The chemokinetic response to FMLP was unaffected by D-penicillamine (250 μM to 10 mM) while alclofenac (500 μM to 1 mM), salicylic acid (250 μM to 10 mM) and indomethacin (100 μM to 1 mM) caused dose-related inhibition.

Introduction

The effects of anti-inflammatory and antirheumatic drugs on leucocyte migration has been studied using either micropore filter assays (Borel, 1973; Rivkin, Foschi & Rosen, 1976; Mowat, 1978) or migration of leucocytes from capillary tubes (Meacock & Kitchen, 1976; Brown & Collins, 1977; 1978). Micropore filter assays have a variety of technical disadvantages (Maderazo & Woronick, 1978) and the distinction of drug effects on directed locomotion (chemotaxis) and on stimulated random migration (chemokinesis) requires the use of complicated chequerboard assays (Zigmond & Hirsch, 1973).

Leucocyte migration from capillary tubes, originally developed as an assay for the release of mediators of delayed hypersensitivity (George & Vaughan, 1962), is time consuming, technically difficult and requires large volumes of blood for the preparation of reasonable numbers of packed capillaries. The agarose microdroplet assay, which has been used for studying macrophage inhibition factor (Harrington & Stastny, 1973) and leucocyte inhibitory factor (McCoy, Dean & Herberman, 1976), appeared to offer many advantages in terms of ease of operation and the number of samples which could be assayed simultaneously.

Chemokinesis is a form of stimulated locomotion considered to be of some importance *in vivo* (Wilkinson, 1978). An *in vitro* model which allows the study of only chemokinesis and the effects of drugs on it would therefore be of interest. This paper describes the adaptation of the agarose microdroplet leucocyte migration inhibition assay as an alternative to established models for studying human polymorphonuclear leucocyte (PMN) chemokinesis. Technical modifications of the method to reduce variation of droplet size and ease evaluation of results have recently been described (Weese, McCoy, Dean, Ortaldo, Burke & Herberman, 1978). The effects of a variety of anti-inflammatory and antirheumatic drugs and other materials on PMN chemokinesis have been examined using this model.

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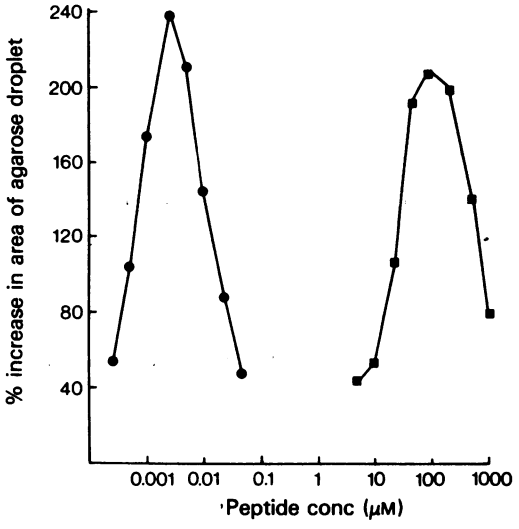

Figure 1 Polymorphonuclear leucocyte (PMN) response to increasing concentrations of FMLP (●) and FMP (■). Points represent the mean percentage increase in area measured in eight replicate wells after 4 h incubation. Similar results were obtained on three other occasions.

Methods

Preparation of cell populations

Mixed peripheral blood leucocytes (70 to 80% PMN) were collected by dextran sedimentation of heparinized venous blood as described previously (Walker, Smith & James, 1979). After removal of remaining erythrocytes by hypotonic lysis, leucocyte populations were washed once with Eagle's minimal essential medium (MEM, Wellcome Reagents), buffered to pH 7.3 with 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES). After centrifugation (150 *g*, 15 min) the cells were resuspended in a small volume (0.15 to 0.2 ml per 20 ml of whole blood) of buffered MEM and allowed to equilibrate at 37°C for at least 10 min. Where possible the same cell source was used to try to eliminate cell variability, slightly different results being obtained with cells from different sources.

Chemokinesins

Two synthetic peptides, formylmethionyl-leucyl-phenylalanine (FMLP) and formyl methionyl-phenylalanine (FMP) (Sigma Chemical Co.), were dissolved in dimethyl sulphoxide at a concentration of 10 mM and diluted with buffered MEM to the required concentrations.

Cell migration assay

Agarose (Indubiose A37) solution (0.8% w/v in distilled water) was allowed to cool to 37°C and mixed with an equal volume of prewarmed filtered 2 × concentrated MEM containing 20% (v/v) heated human serum (56°C, 30 min), 60 mM HEPES, pH 7.3, and 50 units/ml gentamicin. An equal volume of this diluted agarose/MEM solution was added to the leucocyte suspension and thoroughly mixed using a vortex mixer. Samples (2 μl) of the cell suspension (approx. 4×10^5 PMN) were pipetted into each well of cooled 96-well microtitre plates (Sterilin, Ltd.) (20 ml of venous blood provided sufficient cells for two plates which were best prepared separately.) After solidification of the agarose droplets (3 to 5 min) a total volume of 100 μl MEM or peptide, with or without drugs, was added to each well. Up to eight wells were used for each drug concentration, and the first and last series of wells on each plate were MEM controls. Migration was assessed by planimetry of projected areas after 4 h incubation (unless otherwise stated) at 37°C in a humid atmosphere, and expressed as a percentage increase of the area occupied by the agarose droplet.

Drugs

Drugs used in this study were salicylic acid (BDH); indomethacin (Merck, Sharpe & Dohme); D-penicillamine, histamine acid phosphate and vitamin C (Sigma Chemical Co.). Alclofenac and levamisole [(−)-tetramisole] were gifts from Berk Pharmaceuticals and Janssen Pharmaceutica, respectively. Sodium salts were prepared where necessary and all dilutions made in buffered MEM and corrected for any changes in pH. Drug concentrations are expressed as the final concentration of free acid. Human serum albumin (Sigma Chemical Co.) and human fibrinogen (Miles Laboratories) were dissolved in buffered MEM to the required concentrations.

Statistical significance between control and experimental groups was evaluated by Student's *t* test and results were regarded as significant when $P < 0.05$.

Results

Chemokinesis induced by FMLP and FMP

Typical curves obtained to increasing concentrations of FMLP and FMP are shown in Figure 1. EC_{50} values for FMLP and FMP were determined as 650.0 ± 29.0 pM and 27.0 ± 2.4 μM respectively (mean ± s.e. mean of four experiments). Maximum cell movement was similar with both peptides and observed with concentrations of 2.5 nM FMLP and

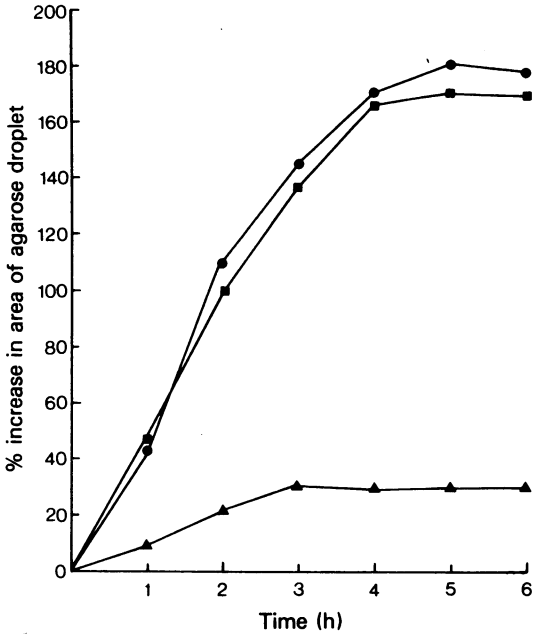

Figure 2 Polymorphonuclear leucocyte (PMN) response to optimal concentrations of FMLP (2.5 nM) (●), FMP (100 μM) (■) and Eagle's minimal essential medium (▲) with time. Points represent the mean percentage increase in area measured in eight replicate wells. Similar results were obtained on two other occasions.

100 μM FMP. The ascending slopes of both curves were parallel and not significantly different by linear regression analysis. Higher concentrations of both peptides inhibited PMN migration.

Table 1 Protein effects on polymorphonuclear leucocyte (PMN) chemokinesis induced by FMLP (2.5 nM)

Protein	*Final percentage concentration*	*% increase in area* *Saline*	*Peptide*
Albumin	0	36.9 ± 2.9	200.9 ± 6.1*
	0.1	64.3 ± 2.5	163.6 ± 6.3*
	1.0	91.9 ± 4.4	149.7 ± 6.0*
	2.5	96.2 ± 3.5	138.8 ± 14.1*
	5.0	97.5 ± 2.5	106.3 ± 2.5
	10.0	102.0 ± 11.4	97.9 ± 3.8
Fibrinogen	0	38.5 ± 1.9	198.7 ± 6.5*
	0.1	46.2 ± 5.0	177.6 ± 9.9*
	1.0	44.1 ± 2.1	138.5 ± 8.8*
	2.5	38.1 ± 1.7	58.2 ± 2.0

Results expressed as mean percentage increase in area ± s.e. mean for eight replicate wells. * $P < 0.01$: results are significantly different from the corresponding saline group.

Figure 2 illustrates the development of cell migration areas with time to optimal concentrations of both FMLP and FMP. Maximum migration was observed at 4 to 5 h with both peptides and with 0.9% w/v NaCl solution (saline) controls.

Reproducibility of the method was determined by repeated day to day measurement of PMN responses to 2.5 nM FMLP. Within individual experiments, the coefficient of variation ranged from 7% to 14%. From day to day the mean increase in area with this concentration of peptide was 150.8 ± 7.2% (mean % change ± s.e. mean, coefficient of variation = 15.4%, $n = 13$). Further experiments (data not shown) indicated that responses observed with purified PMN populations were similar to those obtained with dextran-sedimented cells.

Effect of protein on the polymorphonuclear leucocyte response to FMLP

Human serum albumin alone stimulated cell migration in concentrations of up to 2.5% (w/v). However, increasing concentrations of albumin antagonized the increased migratory response to FMLP which was completely abolished at protein concentrations exceeding 5% (w/v) (Table 1). Human fibrinogen did not stimulate cell migration *per se* but significantly reduced the PMN response to FMLP in a concentration-dependent manner (Table 1).

Effect of histamine and vitamin C on the polymorphonuclear leucocyte response to FMLP

Both histamine and vitamin C potentiated the PMN chemokinetic response to FMLP in a dose-related fashion (Table 2) but the effect of histamine was reduced at higher concentrations.

Table 2 Potentiation of the polymorphonuclear leucocyte (PMN) chemokinetic response to 1 nM FMLP by histamine and vitamin C

Drug	*Final drug concentration* (μM)	*% potentiation*
Histamine	0.003	12.5 ± 3.0
	0.006	13.9 ± 2.0
	0.01	58.1 ± 3.0*
	0.3	42.8 ± 7.0*
	0.6	15.9 ± 4.0
Vitamin C	0.25	20.9 ± 3.6*
	2.5	47.8 ± 5.0*

Results are expressed as the mean percentage potentiation of the control peptide response ± s.e. mean observed in three experiments. * $P < 0.01$: significant difference between control and experimental results.

Potentiation of FMLP- and FMP-mediated chemokinesis by levamisole

Levamisole (100 nM to 25 μM) potentiated the chemokinetic response to FMLP in a dose-dependent manner (Table 3) and at a final concentration of 500 nM, caused a statistically significant parallel shift to the left of the ascending slopes of dose-response curves to both FMLP and FMP. This potentiation of the peptide response was not observed with higher inhibitory peptide concentrations. Cell migration in the absence of either peptide was also enhanced by 500 nM levamisole.

Effect of other drugs on the polymorphonuclear leucocyte response to FMLP

Salicylic acid, indomethacin and alclofenac inhibited the PMN chemokinetic response to FMLP (Table 4). Minimum effective concentrations were: salicylic acid 250 μM, indomethacin 100 μM, alclofenac 500 μM. D-Penicillamine had no effect on the cell response to peptide at final concentrations of 250 μM to 10 mM.

Table 3 Potentiation by levamisole of the polymorphonuclear leucocyte (PMN) chemokinetic response to 1 nM FMLP

Final concentration of levamisole in well					
2.5 μM	1 μM	500 nM	250 nM	100 nM	50 nM
ND	68.1*	54.2*	19.7*	10.7	3.2
90.4*	74.3*	59.1*	26.4*	15.7*	10.0

Results from two individual experiments, expressed as a percentage potentiation of the control peptide response; six replicate wells per drug concentration. ND = not determined. * $P < 0.01$: results are significantly different from the corresponding control group.

Discussion

This paper describes the adaptation of an agarose microdroplet leucocyte migration inhibition assay for the study of chemokinetic movement of human PMNs.

FMLP and FMP, two synthetic formylated peptides, exhibit both chemokinetic and chemotactic activity in filter assays (Showell, Freer, Zigmond, Schiffmann, Aswanikumar, Corcoran & Becker, 1976). The activity of these peptides as chemokinetic agents closely follows their activity as chemotactic agents in terms of effective concentrations, steepness of dose-response curves and the maximum response reached being similar for both materials (Showell *et al.*, 1976). The inhibition of cell migration by higher peptide concentrations (Figure 1) is of interest since it is these concentrations that induce lysosomal enzyme release (Showell *et al.*, 1976) and cell aggregation (O'Flaherty, Kreutzer, Showell, Vitkauskas, Becker & Ward, 1979). Also at these peptide concentrations, large clumps of cells are seen in the agarose droplet. Whether the migration inhibition is related to one or other of these effects or to the phenomenon of desensitization (O'Flaherty *et al.*, 1979) is not clear.

Wilkinson & Allan (1978) suggested that albumin is essential for the PMN response to low molecular weight chemotaxins. In the present study human PMNs responded to FMLP and FMP without the addition of protein, apart from that present in the agarose droplet (approximately 6 ng of albumin). However, the cells were washed only twice before use and substantial quantities of protein, particularly albumin, may still have been associated with the cell membrane (Repine & Clawson, 1978). The small quantity of albumin present and its dilution in the total volume of the well may partially account for the slowing of migration after 4 h. Addition of albumin resulted in an increased random migration confirming the findings of Wilkinson & Allan (1978) but not their suggestion that albumin facilitates the effect of low

Table 4 Effect of antirheumatic drugs on the polymorphonuclear leucocyte (PMN) chemokinetic response to 1 nM FMLP

	Final concentration of drug in well						
Drug	10 mM	5 mM	1 mM	500 μM	250 μM	100 μM	50 μM
Salicylate	98 ± 4*	95 ± 6*	88 ± 3*	56 ± 4*	20 ± 1*	−3 ± 3	ND
Indomethacin	ND	ND	86 ± 3*	56 ± 4*	46 ± 4*	22 ± 2*	−7 ± 1
Alclofenac	ND	ND	89 ± 6*	33 ± 3*	12 ± 1	0	0
D-Penicillamine	2	5	3	0	1	ND	ND

Results expressed as mean percentage inhibition ± s.e. mean relative to control peptide response. Experiments were repeated twice with the exception of D-penicillamine which was studied in only one experiment. At least six replicate wells per drug concentration in each experiment. ND = not determined. * $P < 0.01$: results are significantly different from corresponding control group.

molecular weight peptides by acting as a carrier, since increasing concentrations of albumin (and fibrinogen) antagonized the response to an optimal concentration of FMLP (Table 1). Antagonism of peptide-induced PMN migration by similar concentrations of albumin has been reported by Mukherjee & Lynn (1978) using a micropore filter assay; the antagonism was regarded as competitive since it could be overcome by increasing the peptide concentration.

The potentiation of the PMN response to FMLP by histamine and vitamin C provides further evidence that the agarose micro-droplet method could provide an alternative to the established methods. Anderson, Glover & Rabson (1977), and Goetzl, Wasserman, Gigli & Austin (1974), using micropore filter methods, have shown both materials to potentiate chemokinetic responses. The bell-shaped dose-response curve observed with histamine was similar to that reported by Anderson *et al.* (1977) using endotoxin activated serum as the chemokinesin. The method has also been used to confirm the potentiating effect of levamisole on random and directed migration of human PMN (Anderson, Glover, Koornhof & Rabson, 1976; Wright, Kirkpatrick & Gallin, 1977; Mowat, 1978) and rat peripheral PMN (Cunningham, Ford-Hutchinson, Oliver, Smith & Walker, 1978). These effects had been observed by other techniques.

Chwalinska-Sadowska & Baum (1976) and Mowat (1978) found that D-penicillamine inhibited human PMN chemotaxis after 1 h preincubation. Without preincubation, we found no inhibition in agreement with observations by Mowat (1978).

The inhibition of PMN chemokinesis by salicylic acid and indomethacin was achieved at concentrations similar to those used by Brown & Collins (1978), but the inhibition was more extensive. This discrepancy may be explained at least in part, by the relative lack of protein in the agarose micro-droplet system compared to the capillary tube method, which includes 10% horse serum. Competitive antagonism of the inhibitory actions of several anti-inflammatory drugs by increasing bovine serum albumin concentrations has been demonstrated (Rivkin, 1977). Alclofenac, a non-steroidal, acidic analgesic and anti-inflammatory drug (Lambelin, Roba, Gillet & Buu-Hoi, 1970) also inhibited chemokinetic migration.

In conclusion, a reproducible and reliable method for evaluating the effects of physiological, pathological and synthetic compounds on PMN chemokinetic responses has been described. The method is cheap, large numbers of replicates may be performed using relatively small volumes of blood and evaluation is rapid and straightforward. The effects of a variety of materials obtained with this method are similar to those obtained by the use of more cumbersome, technically difficult procedures.

J.R.W. was the Cowburn Scholar at King's College Hospital Medical School during the course of this study. Correspondence to J.R.W., please

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