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Chemotactic factor receptors of human PMN leucocytes

I. EFFECTS ON MIGRATION OF LABELLING PLASMA MEMBRANE DETERMINANTS WITH IMPERMEANT COVALENT REAGENTS AND INHIBITION OF LABELLING BY CHEMOTACTIC FACTORS

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Summary. The mechanism of stimulation of human PMN leucocyte-directed migration by chemotactic factors was studied by pre-labelling plasma membrane determinants with impermeant covalent reagents and assessing the effects of such modification on spontaneous mitration and chemotaxis in modified Boyden chambers. Pre-treatment of PMN leucocytes with 10^{-9} to 10^{-6} M isethionyl acetimidate, which selectively labels amino groups, enhanced spontaneous migration and concomitantly inhibited chemotaxis to fragments of the fifth component of complement (C5fr), 12-L-OH-5,8,10,14-eicosatetraenoic acid (HETE) and several formyl-methionyl (f-Met) peptides to an extent that was inversely related to the magnitude of the chemotactic response of untreated PMN leucocytes. Para-chloromercuribenzene sulphonate, which selectively labels sulphydryl groups, inhibited chemotaxis to diverse stimuli without substantially influencing spontaneous migration, while the diazonium salt of sulphanilic acid, which labels several types of plasma membrane determinants, altered neither spontaneous nor chemotactic migration. Incubation of PMN leucocytes with various concentrations of [^{3}H]-isethionyl acetimidate labelled from 33,000 amino groups per PMN leucocyte at 10^{-6} M to over 800,000 at 10^{-4} M, a reaction that was substantially inhibited by chemotactic concentrations of C5fr and HETE, but not by f-Met peptides. Subcellular fractionation of PMN leucocytes labelled with [^{3}H]-isethionyl acetimidate localized the radioactivity to membrane-rich fractions. Free amino groups thus appear to be functionally critical determinants of some chemotactic factor receptors on the plasma membrane of PMN leucocytes.

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INTRODUCTION

That the stimulation of polymorphonuclear (PMN) leucocyte-directed migration involves a specific interaction of chemotactic factors with cellular receptors has been inferred from the results of both functional and binding studies with highly purified or synthetic principles. The chemotactic activity of two hydroxy-fatty acid metabolites of arachidonic acid (Goetzl & Gorman, 1978; Goetzl, Woods & Gorman, 1977; Turner, Tainer & Lynn, 1975) and the dependence of the chemotactic activity of several peptide stimuli on the presence and position of hydrophobic amino acid constituents (Goetzl & Austen, 1976; Showell, Freer, Zigmond, Schiffman, Aswanikumar, Corcoran &

Becker, 1976) have suggested that the PMN leucocyte receptors contain critical hydrophobic domains (Boswell, Goetzl & Austen, 1976; Goetzl & Austen, 1976; Goetzl & Gorman, 1978; Showell *et al.*, 1976; Wilkinson, 1977). Functional studies with substituents of the tetrapeptides of the eosinophil chemotactic factor of anaphylaxis (ECF–A) have shown that the hydrophobic amino-terminal tripeptide reversibly inhibits the eosinophil chemotactic activity of the parent tetrapeptide by approximately 50% at equimolar concentrations, implying that they compete for the same hydrophobic portion of the receptors (Boswell *et al.*, 1976; Goetzl & Austen, 1976). The binding of the synthetic chemotactic factor [^{3}H]-formyl-norleucyl-leucyl-phenylalanine to rabbit PMN leucocytes (Aswanikumar, Corcoran & Schiffman, 1977) and of [^{3}H]-formyl-methionyl-leucyl-phenylalanine to human neutrophils (Williams, Snyderman, Pike & Leftkowitz, 1977) is rapid, saturable and rapidly reversible, and is competitively inhibited by other formylated peptides in a rank-order that reflects their chemotactic potency. The specificity of the putative receptor for formylated peptides was further demonstrated by the inhibition of binding of the labelled peptide to rabbit PMN leucocytes by small peptides of bacterial origin, while comparably chemotactic concentrations of the minor fragment of the fifth component of complement, C5a, exhibited no competitive effect (Aswanikumar *et al.*, 1977).

Chemotactic principles alter the properties of PMN leucocyte membranes in terms of net charge and transmembrane potential (Gallin, Durocher & Kaplan, 1975), plasma membrane deformability (Miller, 1978), and rates of flux of some ions (Gallin & Rosenthal, 1974), but the relationships of these events to the stimulation of directed migration have not been elucidated. The structural characteristics that enable bound chemotactic factors to perturb the PMN leucocyte plasma membrane may include a net negative charge (Boswell *et al.*, 1976; Goetzl & Austen, 1976; Goetzl & Gorman, 1978; Wilkinson, 1977) and a susceptibility to rapid proteolytic cleavage by leucocyte ecto-enzymes (Aswanikumar, Schiffman, Corcoran & Wahl, 1976). While the evolving evidence thus supports both the specificity and the plasma membrane localization of the chemotactic factor receptor of PMN leucocytes, the structure of the receptor has not been approached. The effects on chemotaxis of prelabelling human PMN leucocytes with impermeant covalent reagents and the ability of chemotactic factors to prevent such labelling were examined in order to identify plasma membrane constituents of chemotactic factor receptors (Goetzl, Hoe & Smith, 1978). This dual approach has demonstrated that some amino and sulphydryl groups are critical to the chemotactic response of PMN leucocytes and that the plasma membrane amino groups contribute to the specificity of chemotactic factor receptors.

MATERIALS AND METHODS

Disposable polystyrene chemotactic chambers (Adaps, Inc., Dedham, MA) were assembled with micropore filters of 3 μm pore diameter (Sartorius, Göttingen, West Germany) as previously described (Goetzl & Austen, 1972). Hanks's balanced salt solution (Microbiological Associates, Bethesda, MD), dithiothreitol and ovalbumin recrystallized five times (Miles Laboratories, Elkhart, IN), re-crystallized human serum albumin (Schwarz-Mann Div., Becton, Dickinson and Co., Orangeburg, NY), dextran, Sephadex G-75, Sepharose-2B and Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, NJ), sodium diatrizoate (Hypaque, Winthrop Laboratories, New York, NY), disodium ethylenediamine tetra-acetate (EDTA), ascorbic acid, sucrose, ammonium molybdate, trichloroacetic acid, tris(hydroxymethyl) aminomethane and certified grade isobutanol (Fisher Scientific Co., Medford, MA), adenosine 5′-triphosphate (ATP), arachidonic acid, zymosan A and reagent kits for quantifying lactic acid dehydrogenase and β-glucuronidase (Sigma Chemical Co., St Louis, MO), rabbit anti-human C5 (a gift from Dr Hans J. Müller-Eberhard, Scripps Clinic and Research Foundation, La Jolla, CA), DNase (Calbiochem, San Diego, CA) and [γ-^{32}P]-ATP (New England Nuclear, Boston, MA) were obtained as noted. The reagents employed in the labelling of PMN leucocytes or for the synthesis of such labels included methyl-4-mercapto-butyrimidate hydrochloride (ICN Pharmaceuticals, Inc., Cleveland, OH), sulphanilic acid (Aldrich Chemical Co., Inc., Milwaukee, WI), isethionic acid (Eastman Kodak Co., Rochester, NY), acetonitrile (Burdick and Jackson Laboratories, Inc., Muskegon, MI), 2-mercaptoethanol (Calbiochem, San Diego, CA), hydrogen peroxide, 30% solution (Mallinckrodt Inc., St Louis, MO), and *p*-chloromercuribenzene sulphonate (Sigma Chemical Co., St Louis, MO). All other organic solvents were either reagent grade (Eastman Kodak Co., Rochester, NY) or Fisher-certified (Fisher Scientific Co., Medford, MA) and were redistilled before use.

Assessment of PMN leucocyte spontaneous migration and chemotaxis

Blood from normal donors was collected and incubated with citrate anti-coagulant and dextran to sediment the erythrocytes (Goetzl & Austen, 1972). The PMN leucocytes in the supernatant plasma were purified by centrifugation through Ficoll–Hypaque cushions (Boyum, 1968), washed and resuspended in Hanks's balanced salt solution made 0·4 g/100 ml in ovalbumin and 0·005 M in Tris–HCl, pH 7·4 (HBSS–OA) at a standardized concentration of $2{\cdot}0 \pm 0{\cdot}5 \times 10^6$ PMN leucocytes/ml (Goetzl & Austen, 1972; Goetzl & Gorman, 1978). Modified Boyden chambers containing a chemotactic stimulus and those included to allow quantitation of the background migration in the absence of a stimulus were incubated for 2 h at 37°, while chambers employed to assess spontaneous migration were incubated for 3 h at 37°. Processing of filters was as described (Goetzl & Austen, 1972; Goetzl & Gorman, 1978). PMN leucocytes were enumerated microscopically in ten high power fields (hpf), five from each of duplicate filters, at a depth of 80–100 μm from the cell source. The depth in the filters was selected to achieve a background count of three to eight PMN leucocytes per hpf in chemotactic experiments and a level of spontaneous migration of ten to fifteen PMN leucocytes per hpf for untreated cells. Leucocytes were then counted at those levels in experimental and additional control filters without knowledge of the protocol. Spontaneous migration is expressed as PMN leucocytes/hpf and chemotactic responses are expressed as net PMN leucocytes/hpf after subtraction of the background migration in control chambers. The altered spontaneous and chemotactic migration of PMN leucocytes pre-treated with a labelling reagent is denoted as a percentage of the migration of replicate portions of PMN leucocytes pre-treated with buffer alone. Statistical analyses were performed with a standard two-sample Student's *t* test.

Chemotactic fragments of the fifth component of complement (C5fr) were prepared by incubating 20 ml of fresh human serum for 20 min at 37° with 30 mg of zymosan particles that previously had been boiled for 15 min in distilled water and washed twice in Hanks's solution. The supernatant plasma obtained by centrifugation was filtered on a $4{\cdot}0 \times 100$ cm Sephadex G-75 column that was equilibrated and developed with 0·13 M NaCl–0·02 M Na acetate (pH 5·0). The elution volume of the chemotactically active C5fr indicated an apparent mol. wt of 16,800, which corresponds to that of the expected C5a des-Arg product (Fernandez, Henson, Otani & Hugli, 1978). This peak of activity was pooled and concentrated to 10 ml in order to serve as a standard preparation of C5fr. Over 95% of the chemotactic activity in 100 μl of the C5fr bound to a 5 ml bed volume column of Sepharose 2B coupled with monospecific rabbit anti-human C5 (Cuatrecasas, Wilchek & Anfinsen, 1968), that was equilibrated in 0·05 M Tris–0·1 M NaCl buffer (pH 8·0), while no activity bound to Sepharose 2B coupled with normal rabbit serum. 12-L-OH-5,8,10,14-eicosatetraenoic acid (HETE) was prepared as described (Goetzl & Gorman, 1978; Goetzl *et al.*, 1977) by the action of human platelet lipoxygenase on arachidonic acid in the presence of 0·10 mM indomethacin. The product was purified by silicic acid chromatography and its purity confirmed by thin-layer chromatography and gas-liquid chromatography–mass spectrometry (Goetzl & Gorman, 1978; Goetzl *et al.*, 1977). Purified synthetic formyl–methionyl (f–Met) peptides were supplied by Dr R. J. Freer (Medical College of Virginia, Richmond, VA).

Synthesis of impermeant covalent reagents and labelling of PMN leucocytes

Isethionyl acetimidate was synthesized by a modification of the previously described method (Whiteley & Berg, 1974). Isethionic acid was recrystallized from *n*-hexane:toluene (10:1, v:v). Fifty millilitres of acetonitrile containing 1·95 g of isethionic acid were cooled to 0°, 1·95 g of dry HCl gas was added with stirring, and the reactants were incubated for 36 h at 4°. The product was collected by filtration and washed twice in 2 ml of tetrahydrofuran; its melting point was identical to published results (Whiteley & Berg, 1974). The method was modified to synthesize [^{3}H]-isethionyl acetimidate by employing [^{3}H]-acetonitrile and 1/50 the quantities of starting materials (Custom Synthesis Division, New England Nuclear, Boston, MA). The crude radioactive product was purified by silica gel H thin-layer chromatography (Analtek, Newark, DE) with a solvent system composed of ethyl acetate:-methanol:pyridine (20:20:1, v:v:v). Nuclear magnetic resonance spectroscopy (Varian Instruments Division, Palo Alto, CA, model HFT-80) was performed using deuterated water as the solvent and tetramethylsilane as the internal standard. The nuclear magnetic resonance spectrum of the non-radioactive isethionyl acetimidate was identical to the published spectrum (Whiteley & Berg, 1974), while that of the [^{3}H-methyl]-isethionyl acetimidate ([^{3}H]-isethionyl

acetimidate) indicated the presence of a 25–30% contamination with free isethionate. The corrected specific activity of the partially purified compound was 137 mCi/mmol. The diazonium salt of sulphanilic acid was synthesized by the indirect method (Berg, 1969; DePierre & Karnovsky, 1974). The reaction in triplicate of a 1 mM solution of diazotized sulphanilic acid with an equimolar quantity of [^{3}H]-tyrosine (New England Nuclear, Boston, MA) for 30 min at 37° yielded a mean derivatization of 38% of the tyrosine as assessed by thin-layer chromatography.

PMN leucocytes were reacted with non-radioactive or [^{3}H]-isethionyl acetimidate for varying times at 37° in a buffer consisting of 0·135 M NaCl, 0·001 M KH_2PO_4, 0·009 M Na_2HPO_4 and 10 mM glucose, that was adjusted to pH 8·0 by the dropwise addition of 1 M $NaHCO_3$. The diazonium salt of sulphanilic acid was dissolved in 20 ml of Hanks's solution with 10 mM glucose that was titrated to pH 7·4 with 1 M $NaHCO_3$ and incubated with PMN leucocytes in the same buffer for 30 min at 37°. PMN leucocytes were reacted with *p*-chloromercuribenzene sulphonate for 20 or 60 min at room temperature in a buffer consisting of 0·13 M NaCl, 0·003 M Na_2HPO_4, 0·002 M KH_2PO_4, 10 mM glucose and 5 mM $CaCl_2$ (pH 7·0). The reaction of PMN leucocytes with methyl-4-mercaptobutyrimidate involved a two stage protocol (Traut, Bollen, Sun, Hershey, Sundberg & Pierce, 1973). The butyrimidation reaction was carried out for 15–60 min at 4° in Hanks's solution with 10 mM glucose, 7 mM β-mercaptoethanol to minimize disulphide bond formation, and sufficient 1 M $NaHCO_3$ to adjust the pH to 8·0. The leucocytes were washed three times and resuspended in Hanks's solution made 1 mM in H_2O_2 followed by an additional incubation at room temperature for 30 min to allow the formation of disulphide bonds. The concentration range of PMN leucocytes was $3·0–4·5 \times 10^7$/ml for all labelling reactions, and the leucocytes were washed three times in Hanks's solution with 0·005 M Tris–HCl and 0·2 g/100 ml of ovalbumin (pH 7·4) prior to assessment of spontaneous and chemotactic migration. None of the reagents resulted in cell aggregation or lysis, and viability always exceeded 95% as assessed by the exclusion of trypan blue dye (Allied Chemical Corp, New York, NY).

Subcellular fractionation of [^{3}H]-isethionyl acetimidate-labelled PMN leucocytes

As it has been noted that heavy labelling with isethionyl acetimidate leads to extensive aggregation of membranes (Whiteley & Berg, 1974), procedures for the isolation of subcellular fractions from labelled PMN leucocytes were modified accordingly. Two to three $\times 10^8$ PMN leucocytes that previously had been labelled with [^{3}H]-isethionyl acetimidate were washed three times with calcium and magnesium-free Hanks's solution containing 2 mM EDTA and 0·005 M Tris–HCl (pH 7·4), and were resuspended in 1 ml of 0·10 M sucrose that had been adjusted to pH 7·0 with 1 M $NaHCO_3$ after the addition of 50 u/ml of DNase, 0·01% β-mercaptoethanol, and EDTA and Tris at the concentrations noted. The PMN leucocytes were disrupted at 4° in a glass tube with 100 strokes of a Teflon homogenization pestle (Tri-R Instruments, Inc., Rockville Center, NY) operated at 6000 r.p.m. Six hundred and sixty microlitres of 0·70 M sucrose were added to restore isotonicity and an additional 340 μl of 0·34 M sucrose was used to rinse the pestle and homogenization tube. Two hundred microlitres of the homogenate were saved for analysis and the remainder was centrifuged at 100 ***g*** for 20 min at 4°. The 100 ***g*** supernatant was centrifuged at 400 ***g*** for 20 min at 4° and the 400 ***g*** supernatant was layered on a 10 ml linear gradient of 10–50% (g/100 ml) sucrose, containing 5 mM EDTA and 0·01% (v/v) β-mercaptoethanol, that was centrifuged at 20,000 ***g*** for 45 min at 4°. The gradient was harvested in twenty 590 μl fractions and the 100 ***g*** and 400 ***g*** pellets were resuspended in 1 ml of 0·34 M sucrose with EDTA, Tris and β-mercaptoethanol. All fractions were vigorously mixed, a portion was removed for fixation and electron microscopic examination, and the remainder of each was sonicated for 5 min at 4° employing 200 watts in a continuous mode (Branson Sonic Power Co., Danbury, CT). Two-thirds of the sonicated 100 ***g*** pellet was applied to a second linear gradient of 10–50% (g/100 ml) sucrose which was centrifuged at 20,000 ***g*** for 40 min at 4°. The second gradient was harvested in three fractions including the top 2·0 ml, which contained the volume of the original homogenate or sonicate and the region of the interface with the sucrose gradient, an opalescent band of approximately 2 ml in the upper one-half of the gradient, and the pellet which was resuspended in 2 ml of 0·34 M sucrose-5 mM EDTA and re-sonicated as noted above. Portions of all fractions were assayed for the lysosomal marker (Walsh & Spitznagel, 1971) β-glucuronidase (Fishman, 1963), the cytoplasmic marker (Welsh & Spitznagel, 1971) LDH (Kornberg, 1955), and the plasma-membrane marker (Harlan, DeChatalet, Iverson & McCall, 1977) magnesium-dependent ATPase (DePierre & Karnovsky, 1973). For the latter assay, [γ-^{32}P]-ATP was purified within

24 h prior to use by PEI-cellulose thin-layer chromatography as described (Vansteveninck, Weed & Rothstein, 1965). By definition, one unit of β-glucuronidase liberated 1 μg of phenolphthalein from phenolphthalein glucuronide per hour, one unit of LDH generated 1 μmol of NADH per 30 min, and one unit of ATPase cleaved one pmol of [γ-^{32}P]-ATP per hour in the presence of a twenty-fold molar excess of *p*-nitrophenyl phosphate.

RESULTS

Effects of impermeant labelling reagents with differing specificities on human PMN leucocyte spontaneous and chemotactic migration

Isethionyl acetimidate, which selectively derivatizes amino groups on the plasma membrane of intact cells (Whiteley & Berg, 1974), enhanced spontaneous migration and concomitantly inhibited chemotaxis in a dose-related fashion (Fig. 1). The stimulation of spontaneous migration achieved a plateau at concentrations of 10^{-8} to 10^{-6} M isethionyl acetimidate. The chemotactic responses to optimal levels of C5fr and F-Met-Phe-Leu were significantly inhibited by pretreatment with isethionyl acetimidate at concentrations of 10^{-9} to 10^{-6} M and 10^{-6} M, respectively. In three additional experiments, 10^{-8} M isethionyl acetimidate inhibited the chemotactic response to 10 μg/ml of HETE by 49 ± 11% (mean ± SD), to 10^{-7} M F-Met-Leu-Ala-Phe by 43 ± 9%, and to 10^{-7} M F-Met-Leu-Glu by 39 ± 12%. The time-course of the effects of prelabelling PMN leucocytes with isethionyl acetimidate was examined by introducing a single labelling dose at the onset of the period of incubation and then for some replicates adding one to three more labelling doses at 30 min intervals (Fig. 2). The latter interval was employed as isethionyl acetimidate is hydrolysed at pH 8·0 with a $t_{\frac{1}{2}}$ of approximately 20 min (Whiteley & Berg, 1974). Incubation with one dose of 10^{-8} M isethionyl acetimidate stimulated spontaneous migration at 30 and 60 min, and suppressed the chemotactic responses to C5fr and F-Met-Phe-Leu for 30–90 min. The effects of the single dose on spontaneous migration and chemotaxis subsided by 90 min and 120 min, respectively. Multiple additions of isethionyl acetimidate enhanced spontaneous migration and suppressed the chemotactic responses to C5fr and F-Met-Phe-Leu to a significantly greater extent than a single labelling dose at 90 and 120 min (Fig. 2). The chemo-

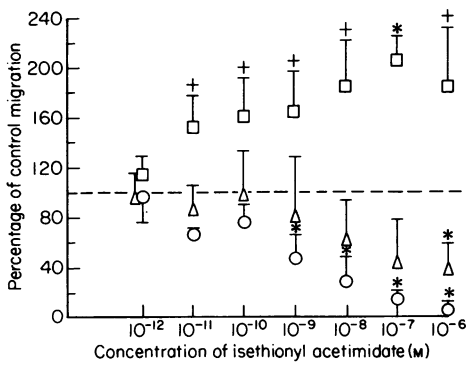

Figure 1. Effects of isethionyl acetimidate on human PMN leucocyte migration. Portions of PMN leucocytes were incubated for 30 min at 37° with various concentrations of isethionyl acetimidate. The results are shown as the mean ± SD for three experiments. The spontaneous migration (□) of control PMN leucocytes (100%) was 14·6, 16·3, and 18.9 leucocytes/hpf while the chemotactic responses (100%) to 5×10^{-8} M F-Met-Phe-Leu (△) were 37·6, 27·8, and 23·4 net leucocytes/hpf and to a 1/80 dilution of C5fr (○) were 26·9, 31·4, and 33·7 net leucocytes/hpf in the triplicate experiments. Levels of statistical significance in comparison to control values are: + = $P < 0.05$ and * = $P < 0.01$.

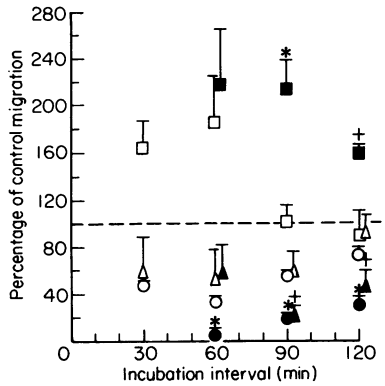

Figure 2. Time course of isethionyl acetimidate effects on PMN leucocyte migration. Portions of PMN leucocytes were incubated for intervals varying from 30 to 120 min with 10^{-8} M isethionyl acetimidate added at time zero min and for some replicates again at times 30, 60 and/or 90 min. The results are shown as the mean ± SD for three experiments. Control PMN leucocytes exhibited levels of spontaneous migration (squares) (100%) of 12·6, 13·1, and 15·7 leucocytes/hpf and the chemotactic responses (100%) to 5×10^{-8} M F-Met-Phe-Leu (triangles) were 20·9, 28·7 and 31·4 and to a 1/80 dilution of C5fr (circles) were 23·9, 28·1, and 32·6 net leucocytes/hpf in triplicate experiments. Levels of statistical significance are depicted as in Fig. 1, but represent a comparison of the effects of multiple additions (closed symbols) to the effects of a single dose (open symbols) of reagent.

tactic response to C5fr also was more significantly inhibited at 60 min by two labelling doses as compared to one. The relationship of the extent of inhibition of chemotaxis by isethionyl acetimidate to the magnitude of the chemotactic response of untreated PMN leucocytes was studied by quantifying the responses of labelled PMN leucocytes to a range of concentrations of C5fr, F-Met-Phe-Leu and HETE (Fig. 3). Regardless of the stimulus, the percentage inhibition of chemotaxis was inversely proportional to the magnitude

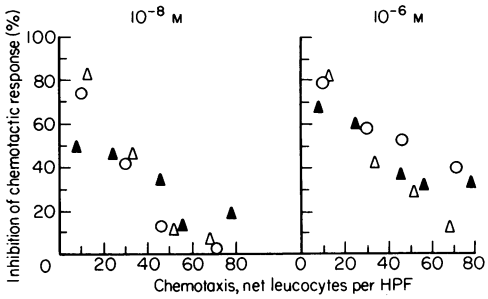

Figure 3. The relationship of the magnitude of the chemotactic response to the chemotactic inhibitory effect of isethionyl acetimidate. Portions of PMN leucocytes were preincubated for 30 min at 37° with one of two concentrations of isethionyl acetimidate. The increasing concentrations of chemotactic stimuli which evoked the control responses (0% inhibition) were: 1/160, 1/80, 1/40 and 1/20 dilutions of C5 fragments (○); 10^{-8} M, 3×10^{-8} M, 10^{-7} M, 3×10^{-7} M, and 10^{-6} M F-Met-Phe-Leu (△); and 1, 2, 5, 10, and 20 μg/ml of HETE (▲).

of the response of unlabelled PMN leucocytes and 50% inhibition by 10^{-8} and 10^{-6} M isethionyl acetimidate occurred at levels of chemotaxis of approximately twenty-five and forty net leucocytes/hpf, respectively. The specificity of the chemotactic inhibition was suggested by the fact that labelling with 10^{-6} M isethionyl acetimidate failed to prevent the stimulation of PMN leucocyte chemokinesis by 2 mM sodium ascorbate in three experiments.

PMN leucocytes were preincubated for 20 min at room temperature with a wide range of concentrations of *p*-chloromercuribenzene sulphonate, which derivatizes sulphydryl groups on the plasma membrane (Vansteveninck *et al.*, 1965), and then were washed prior to assessing their spontaneous migration and chemotactic responses (Fig. 4). The chemotactic response to F-Met-Phe-Leu was significantly inhibited by 10^{-10} to 10^{-5} M *p*-chloromercuribenzene sulphonate to an extent that was proportional to the labelling concentration, while spontaneous migration

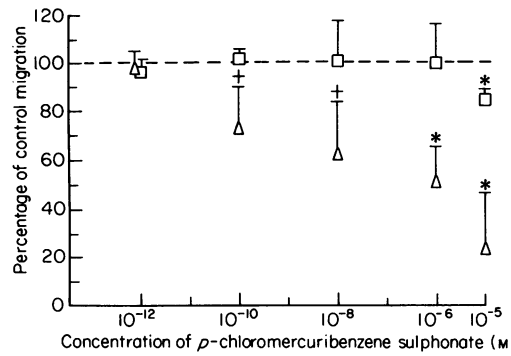

Figure 4. Effects of *p*-chloromercuribenzene sulphonate on human PMN leucocyte migration. Portions of PMN leucocytes were incubated for 20 min at 23° with various concentrations of *p*-chloromercuribenzene sulphonate. The results depicted are the mean ± SD for three experiments. Control PMN leucocytes exhibited levels of spontaneous migration (□) (100%) of 10·7, 13·3 and 12·6 leucocytes/hpf, while the chemotactic responses (△) (100%) to 5×10^{-8} M F-Met-Phe-Leu were 19·8, 27·1, and 34·6 net leucocytes/hpf in the triplicate experiments. Levels of statistical significance are depicted as in Fig. 1.

was only slightly inhibited at the highest concentration of 10^{-5} M. In three comparable experiments, pretreatment of PMN leucocytes with 10^{-6} M *p*-chloromercuribenzene sulphonate inhibited chemotaxis to a 1/80 dilution of C5fr by 69 ± 13%, to 10 μg/ml of HETE by 61 ± 17% (mean ± SD), and to 10^{-7} M F-Met-Leu-Glu by 48 ± 10%. As derivatization of both amino and sulphydryl groups on the plasma membrane significantly altered PMN leucocyte migration, the bifunctional reagent methyl-4-mercapto-butyrimidate was studied in view of its capacity to bind to both types of groups concurrently (Traut *et al.*, 1973). The two-stage pre-labelling protocol consisted of butyrimidation of the PMN leucocyte plasma membrane amino-groups in the presence of 7 mM β-mercaptoethanol to prevent disulphide bond formation, followed by washing the cells and incubation in 1 mM hydrogen peroxide to facilitate the reaction of the mercapto-portion of the bound reagent with leucocyte membrane sulphydryl groups (Fig. 5). The effects observed were analogous to those achieved by pre-labelling with isethionyl acetimidate, as 3×10^{-4} M and 6×10^{-4} M methyl-4-mercaptobutyrimidate significantly enhanced the spontaneous migration of PMN leucocytes, while concentrations of 3×10^{-4} M to $1{\cdot}2 \times 10^{-3}$ M significantly inhibited the chemotactic responses to both C5fr and F-Met-Phe-Leu. In three additional experiments, the effects of pre-labelling with 6×10^{-4} M methyl-4-mer-

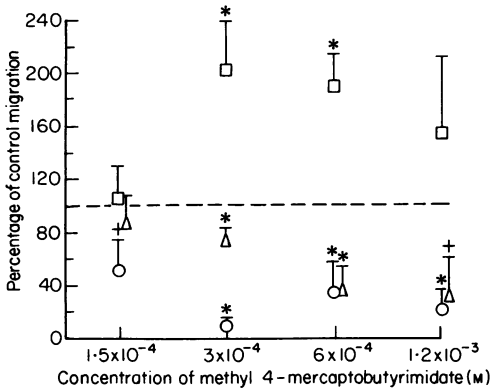

Figure 5. Effects of methyl-4-mercaptobutyrimidate on human PMN leucocyte migration. Portions of PMN leucocytes were incubated for 15 min at 4° with various concentrations of methyl-4-mercaptobutyrimidate and 7 mM β-mercaptoethanol, washed and incubated for 30 min at 23° with 1 mM hydrogen peroxide as described under Methods. The results depicted are the mean $\pm$SD for three experiments. Control PMN leucocytes were sequentially incubated with β-mercaptoethanol and hydrogen peroxide in the absence of the labelling reagent, and exhibited levels of spontaneous migration (□) (100%) of 9·4, 10·1, and 13·6 leucocytes/hpf, while the chemotactic responses (100%) to C5fr at a 1/80 dilution (○) were 20·9, 23·5 and 27·1 net leucocytes/hpf, and to 5×10^{-8} M F-Met-Phe-Leu (△) were 22·6, 23·9, and 30·7 net leucocytes/hpf in triplicate experiments.

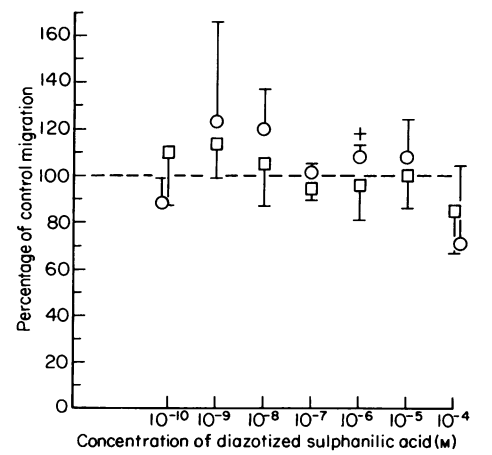

Figure 6. Effects of diazotized sulphanilic acid on PMN leucocyte migration. Portions of PMN leucocytes were incubated for 30 min at 37° with various concentrations of diazotized sulphanilic acid. The results depicted are the mean $\pm$SD for three experiments. Control PMN leucocytes exhibited levels of spontaneous migration (□) (100%) of 12·3, 14·8, and 15·6 leucocytes/hpf, while the chemotactic responses (100%) to C5fr at a 1/80 dilution (○) were 23·8, 28·0 and 34·6 net leucocytes/hpf in triplicate experiments.

captobutyrimidate on spontaneous migration and chemotaxis to C5fr were decreased by a mean of 81% and 69%, respectively, in the absence of β-mercaptoethanol, and 42% and 27%, respectively, with the omission of hydrogen peroxide. In contrast to the effects of the specific amino and sulphydryl group reagents, pre-labelling with the impermeant diazonium salt of sulphanilic acid, which reacts preferentially with hydroxy-phenyl and imidazolyl groups and to lesser extent with amino and sulphydryl groups (Berg, 1969; DePierre & Karnovsky, 1974), did not substantially influence the spontaneous migration of PMN leucocytes nor their chemotactic response to C5fr (Fig. 6), HETE or F-Met-Phe-Leu (not shown).

Labelling of PMN leucocytes with [^{3}H]-isethionyl acetimidate and inhibition of labelling by chemotactic factors.

The effects of isethionyl acetimidate on PMN leucocyte spontaneous migration and chemotaxis to diverse stimuli suggested the possibility that some of the derivatized amino groups were substituents of plasma-membrane receptors for chemotactic factors. In order to further examine the relationships of the isethionyl acetimidate-reactive amino groups to such receptors, portions of $1{\cdot}8$–$2{\cdot}2 \times 10^7$ PMN leucocytes were incubated for 40 min at 37° with various concentrations of [^{3}H]-isethionyl acetimidate in the presence or absence of a chemotactic factor. The labelled cells were washed three times in calcium and magnesium-free HBSS–OA and were dissolved in 2 ml of 3 g/100 ml sodium dodecyl sulphate for quantitation of the cell-associated radioactivity. In the absence of a chemotactic factor, the number of amino groups per PMN leucocyte that were coupled with [^{3}H]-acetamidine increased progressively from approximately 33,000 at 10^{-6} M to over 800,000 at 10^{-4} M [^{3}H]-isethionyl acetimidate (Fig. 7). The presence of HETE or C5fr during the incubation with [^{3}H]-isethionyl acetimidate significantly decreased the number of [^{3}H]-acetamidine derivatives per PMN leucocyte, an effect that was apparent up to the maximum density of labelling. In contrast, a fully chemotactic concentration of F-Met-Leu-Ala-Phe did not prevent the derivatization of amino groups by [^{3}H]-isethionyl acetimidate (Fig. 7). The relationship of the extent of inhibition of labelling by a given concentration of a chemotactic factor to its chemotactic activity was determined for a range of concentrations of structurally diverse principles (Table 1). For C5fr and HETE, the percentage inhibi-

Table 1. Dose–response of inhibition by chemotactic stimuli of [^{3}H]-isethionyl acetimidate labelling of PMN leucocytes*

C5fr				
Stimulus concentration†	1/160	1/80	1/40	1/20
Chemotactic activity‡	9±3	27±5	59±11	83±37
% Inhibition of labelling§	15±3	41±2	59±8	64±6
HETE				
Stimulus concentration	0·25 μg/ml	1 μg/ml	4 μg/ml	16 μg/ml
Chemotactic activity	3±6	14±4	31±9	62±15
% Inhibition of labelling	1±8	11±5	23±3	51±11
F-Met-Leu-Ala-Phe				
Stimulus concentration	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
Chemotactic activity	21±10	38±13	60±17	81±24
%Inhibition of labelling	−14±0	−5±6	−15±7	−51±13
F-Met-Leu-Glu				
Stimulus concentration	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
Chemotactic activity	15±9	26±13	35±12	62±20
Inhibition of labelling	11±8	4±7	2±4	30±9

* The results depicted are the mean ± range of two separate experiments performed in triplicate.

† The concentrations of C5 fragments noted represent the dilution of the standard purified preparation.

‡ The chemotactic responses have been corrected by the subtraction of mean background migration in the two experiments which was 3·7 and 5·1 PMN leucocytes/hpf, respectively, and values have been rounded to the nearest whole number. The numbers presented are net PMN leucocytes/hpf.

§ Labelling of the PMN leucocytes with 3×10^{-5} M [^{3}H]-isethionyl acetimidate in the two experiments gave [^{3}H]-acetamidine counts of 1966 and 2089 DPM/10^7 PMN leucocytes, respectively, in the absence of a chemotactic factor and these values represent 0% inhibition. A negative number indicates an enhancement of labelling by [^{3}H]-isethionyl acetimidate.

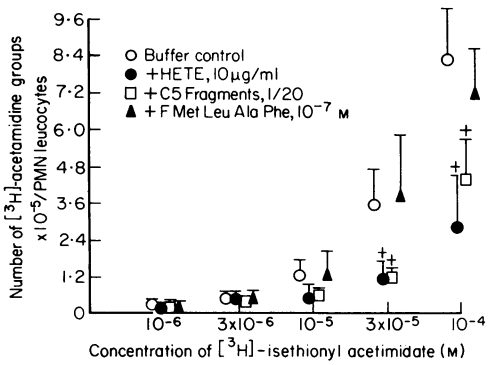

Figure 7. Influence of chemotactic factors on the labelling of PMN leucocytes by [^{3}H]-isethionyl acetimidate. The results shown are the mean ±SD for three experiments. The labelling of 2×10^7 PMN leucocytes yielded levels of radioactivity of 1863±762 d.p.m. (mean±SD) at 10^{-5} M [^{3}H]-isethionyl acetimidate, 5304±1806 d.p.m. at 3×10^{-5} M, and 12,432±2439 d.p.m. at a concentration of 10^{-4} M.

tion of labelling was directly related to the chemotactic activity of the concentration employed in the labelling reaction. In contrast, optimally chemotactic concentrations of the formyl–methionyl peptides failed to inhibit labelling and at the highest concentrations enhanced labelling. Further, the chemotactically inactive agents human serum albumin, at concentrations of 0·1–10 μg/ml, and arachidonic acid, at concentrations of 1–30 μg/ml, failed to influence the degree of labelling observed.

Subcellular localization of the radioactivity in PMN leucocytes labelled with [^{3}H]-isethionyl acetimidate

The subcellular distribution of the [^{3}H]-acetamidine label was examined for PMN leucocytes derivatized in the absence and presence of C5fr (Table 2). Portions of $2–3 \times 10^8$ labelled cells were washed and homogenized in hypotonic sucrose which disrupted more than 98%

Table 2. Subcellular distribution of [^{3}H]-acetamidine label in PMN leucocytes

	Total/10^8 PMN leucocytes (100%)	Fraction (% of total)							
				Sucrose gradient, 400 *g* supernatant			Sucrose gradient, sonicate of 100 *g* pellet‡		
		100 *g* pellet	400 *g* pellet	Lower band	Upper band	Top layer	Lower band	Upper band	Top layer
Enzyme activity*									
Mg^+-ATPase	32·8 units	43	9	7	1	39	17	15	12
β-glucuronidase	19·0 units	2	0	73	2	21	0	0	0
LDH	0·52 units	3	0	0	1	89	0	0	0
[^{3}H]-acetamidine label†									
Control	65,629 ± 9,164	36·7 ± 1·2	5·7 ± 1·1	2·0 ± 0·0	3·3 ± 1·2	40·7 ± 6·7	4·7 ± 3·1	3·7 ± 1·5	21·0 ± 3·6
C5 fragments present during labelling	22,676 ± 5,275+	20·0 ± 3.6+	5·0 ± 1·0	4·7 ± 1·5	6·0 ± 0·4	49·7 ± 10·0	4·3 ± 0·6	5·3 ± 2·1	12·7 ± 0·6

* The results presented are the mean values for the unfractionated homogenates and subcellular fractions of PMN leucocytes from three separate donors that were labelled in the absence of a chemotactic factor. Mean values for PMN leucocytes labelled in the presence of a chemotactic factor were essentially identical.

† C5 fragments were employed at a 1/20 dilution of the standard purified preparation. The results given are the mean ± SD. Statistical significance was calculated by the paired *t* test; *P* values of $<0{\cdot}01$ are noted by the symbol +.

‡ Values shown are referrable to the total enzymatic activity and radiolabel recovered in the 100 *g* pellet, but are expressed as a percentage of the total homogenate.

of the cells. The homogenates were fractionated by sequential centrifugation at 100 *g* and 400 *g*, followed by isopycnic sedimentation of the 400 *g* supernatant in a linear sucrose gradient. Electron microscopic examinations revealed aggregated membranes with free chromatin and some nuclei in the 100 *g* pellet, a fact attributed to the extensive aggregation of membranes labelled with isethionyl acetimidate. The 400 *g* pellet contained nuclei alone, while lysosomal granules were found in the visible lower band of the gradient. Small fragments of membranes were present both in a barely visible upper band and at the top of the gradient in the 0·34 M sucrose from the 400 *g* supernatant. For PMN leucocytes derivatized in the absence of a chemotactic factor, the [^{3}H]-acetamidine label recovered was distributed equally between the membrane-rich 100 *g* pellet and the top of the gradient, in a distribution comparable to the plasma membrane marker magnesium-dependent ATPase. In contrast, little label was found with the nuclei in the 400 *g* pellet or the lysosomal granules in the lower band of the gradient (Table 2). Centrifugation of the top layer of the gradient at 100,000 *g* for 45 min pelleted a mean of 54% of the radioactivity in two of the experiments, confirming the association of the label with the membrane fraction. The presence of C5fr during the labelling reaction not only reduced the total extent of labelling, but also significantly decreased the percentage of this reduced amount that was recovered with the 100 *g* pellet containing the aggregated membranes. Sonication of the 100 *g* pellet and isopycnic sedimentation of the sonicate in a linear sucrose gradient led to the recovery of most of the radioactivity in the top layer, while only approximately 30% of the magnesium-dependent ATPase was in the top layer. Thus, the [^{3}H]-acetamidine label of the aggregated membranes was redistributed by sonication either to small membrane fragments lacking the ATPase marker or to soluble labelled determinants.

DISCUSSION

The demonstration that specific determinants on the plasma membrane are critical to the integrity of a PMN leucocyte receptor for chemotactic factors requires both that alterations in migration occur upon selective derivatization of the determinants and that chemotactic factors competitively block the derivatization. Impermeant covalent reagents which label amino and sulphydryl groups on the plasma membranes of intact cells substantially altered the migration of human PMN leucocytes. Pre-treatment of PMN leucocytes with isethionyl acetimidate, which

selectively derivatizes plasma membrane amino groups, significantly stimulated spontaneous migration in a dose-related fashion that achieved a plateau of two-fold enhancement at concentrations of 10^{-8} to 10^{-6} M (Fig. 1). The chemotaxis of comparably labelled PMN leucocytes was significantly inhibited in a dose–response fashion irrespective of the chemotactic stimulus. *p*-Chloromercuribenzene sulphonate, which selectively labels plasma membrane sulphydryl ryl groups, significantly inhibited the chemotactic response of PMN leucocytes to diverse stimuli in a manner similar to isethionyl acetimidate, but failed to influence spontaneous migration (Fig. 4). In contrast, pre-labelling PMN leucocytes with diazo-sulphanilic acid altered neither form of migration (Fig. 6). The bifunctional reagent, methyl-4-mercaptobutyrimidate, reacts with both amino and sulphydryl groups and was employed in a manner that enabled the label to cross-link the two types of determinants. Labelling the PMN leucocytes with this cross-linking reagent led to effects on migration that were identical to those of isethionyl acetimidate (Fig. 5), which indicates that the derivatization of amino groups predominates functionally over both the labelling of sulphydryl groups and the cross-linking of the two types of groups. The enhancement of spontaneous migration and the inhibition of chemotaxis that resulted from treating PMN leucocytes with a single dose of isethionyl acetimidate persisted for 60–90 min and subsided by 120 min, but both effects could be sustained at significant levels by the repetitive introduction of additional portions of isethionyl acetimidate (Fig. 2). The extent of inhibition of chemotaxis was inversely related to the intensity of chemotactic stimulation (Fig. 3), so that labelling which resulted in 80% inhibition of the response to a threshold concentration of a stimulus frequently failed to influence the response to a maximal concentration of the same stimulus. That the effects of isethionyl acetimidate on PMN leucocyte spontaneous and chemotactic migration were reciprocal in nature and exhibited a self-limited time course, suggested that this reagent was labelling a determinant in a chemotactic factor receptor that was rapidly being restored by the cell.

The labelling of PMN leucocytes with [^{3}H]-isethionyl acetimidate permitted an assessment of the number of derivatized amino groups which may serve as substituents of chemotactic factor receptors and demonstrated that the labelled amino groups were localized on the plasma membranes. The minimal density of labelling that was detectable given the specific activity of the [^{3}H]-isethionyl acetimidate amounted to approximately 33,000 [^{3}H]-acetamidine groups per PMN leucocyte (Fig. 7). The extent of labelling increased progressively with increasing concentrations of [^{3}H]-isethionyl acetimidate to a maximal level of over 800,000 [^{3}H]-acetamidine groups per PMN leucocyte. Structurally diverse chemotactic factors, of which only C5fr possesses free amino groups, specifically inhibited the labelling of PMN leucocytes by [^{3}H]-isethionyl acetimidate. The extent of inhibition of labelling was a function of the concentration of the chemotactic stimuli (Table 1). When examined at comparable levels of PMN leucocyte chemotactic activity, HETE and C5fr inhibited the derivatization of PMN leucocytes to the same extent. That the inhibition of labelling approached 70% (Fig. 7) indicated that a large fraction of the amino groups that are susceptible to derivatization by isethionyl acetimidate interact with the chemotactic factors with a substantial affinity. Subcellular fractionation of PMN leucocytes pre-labelled with [^{3}H]-isethionyl acetimidate led to recovery of over 75% of the total radioactivity in two membrane-rich fractions in association with over 82% of the plasma membrane marker magnesium-dependent ATPase (Table 2). None of the [^{3}H]-acetamidine label was found in the nuclear or lysosomal granular fractions. The finding of much of the [^{3}H]-acetamidine label in a 100 ***g*** pellet containing aggregated plasma membranes from hypotonically lysed and mechanically disrupted PMN leucocytes is consistent with previous observations of the tendency of isethionyl acetamidate labelling to induce extensive aggregation of membranes from other cell types (Whiteley & Berg, 1974), an effect which is partially prevented by the presence of β-mercaptoethanol. While part of the [^{3}H]-acetamidine label in the light membrane and cytosol fraction was sedimented by centrifugation at 100,000 ***g***, a portion amounting to approximately 25–30% of the total radioactivity was soluble. Sonication of the 100 ***g*** membrane pellet redistributed the [^{3}H]-acetamidine label to the top of the sucrose gradient and separated the radiolabel from over 60% of the magnesium-dependent ATPase. The radioactivity in this fraction may be presented in small fragments of membrane or may represent [^{3}H]-acetimidine-derivatized phosphatidyl-serine, a related amino-lipid or a membrane peripheral protein or peptide which has been dispersed into the fluid phase during mechanical disruption of the plasma membrane (Verkleij, Zwaal, Roelofsen, Comfurius, Kastelijn & Van Deenen, 1973).

The possibility that isethionyl acetimidate was labelling plasma membrane amino groups critical to some PMN leucocyte receptors for chemotactic factors was initially raised by the functional effects of such labelling (Figs 1–3) and was supported by the ability of some chemotactic factors to inhibit labelling by [^{3}H]-isethionyl acetimidate to a degree that was proportional to their chemotactic activity (Table 1) and exceeded 2/3 of the total label (Fig. 7). The specificity of the inhibition of labelling by the chemotactic factors was confirmed by the lack of protection afforded by arachidonic acid or human serum albumin. The inability of maximal chemotactic doses of the formyl-methionyl peptides to prevent labelling by [^{3}H]-isethionyl acetimidate superficially appears to support previous data, derived from binding studies (Aswanikumar *et al.*, 1977), which suggested that the PMN leucocyte receptors for formyl-methionyl peptides are distinct from those for the chemotactic fragments of C5. The present observations indicate that the complexity of the interactions of chemotactic factors with PMN leucocytes have not been fully appreciated. Pre-labelling of PMN leucocytes with isethionyl acetamidate inhibited the chemotactic response to the formyl-methionyl peptides with a dose–response (Figs 1 and 3) and time course (Fig. 2) that were comparable to those seen with the chemotactic stimuli which block labelling by [^{3}H]-isethionyl acetimidate. Although the formyl-methionyl peptides failed to inhibit labelling of PMN leucocyte plasma membrane amino groups at concentrations of 10^{-8} to 10^{-6} M, several of these peptides actually enhanced labelling at 10^{-5} M (Table 1). These data together suggest that the formyl-methionyl peptides may interact with the same surface receptors on PMN leucocytes as C5fr and HETE, but that the formyl-methionyl peptides interact differently with one or more amino groups in the receptor. The set of chemotactic factor receptors with isethionyl acetimidate-susceptible amino groups appears to accept numerous chemotactic stimuli, but the specific nature of the occupancy of these receptors may vary with each such principle.

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