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Countergradients of Nonchemotactic Ligands for *N*-Formylmethionylleucylphenylalanine (FMLP) Receptors Promote FMLP-Induced Chemotaxis (42250)

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Abstract. Using the under-agarose chemotaxis assay, addition of compounds known to inhibit chemotaxis of leukocytes toward *N*-f-Met–Leu–Phe (FMLP) was made to the first well in a line of three wells, leukocytes to the middle well, and a slight excess of FMLP to the third well. The compounds included rifampin, fusidic acid, carbobenzoxy Phe–Met, phenylbutazone, sulfinpyrazone, sulfasalazine, and sulfapyridine. The countergradients created in this system markedly stimulated, not inhibited, locomotion toward FMLP. These results, based on functional responses, confirm data using radiolabeled FMLP and support the hypothesis that these compounds are nonchemotactic ligands for FMLP receptors.

Phagocyte receptor pharmacology studies have been greatly facilitated by the discovery of a series of small peptides, exemplified by *N*-f-Met–Leu–Phe (FMLP), that appear to act as specific chemotactic ligands (1, 2). The evidence for the existence of a specific FMLP receptor is based on inhibition of responses to FMLP and binding of radiolabeled FMLP (3–7), the existence of different receptors for other chemotactic ligands (8–10), the isolation of receptor proteins (11–14), and the discovery of nonchemotactic ligands that compete with FMLP for its receptor (15–21).

In the course of studying one of the latter, rifampin, for its effects on under-agarose chemotaxis (16, 20), the following hypothesis was generated. It was reasoned that if excess concentrations of FMLP were present in agarose, so that locomoting leukocytes would be immobilized, then a rifampin gradient moving across the immobilized leukocyte would induce movement. Thus, the advancing rifampin gradient would displace FMLP from "near side" receptors while "far side" receptors would still be occupied by FMLP. The leukocyte would "sense" the artificially induced gradient and move directionally, i.e., chemotactically, away from the rifampin gradient. Attempts to produce this effect were successful in preliminary experiments; however, they were successful in only four of six experiments.

This report describes a procedure which has consistently produced enhanced directed locomotion toward FMLP gradients using countergradients of rifampin as well as a variety of other compounds reported to bind to the FMLP receptor. Countergradients were formed by adding nonchemotactic ligands to the first well in a line of three wells, leukocytes to the middle well, and FMLP to the third well.

Materials and Methods. Sources of chemicals were as follows: from Calbiochem, La Jolla, California, rifampin; Vega Biochemicals, Tucson, Arizona, *N*-formylmethionylleucylphenylalanine (FMLP) and carbobenzoxyphenylalanylmethionine (carbobenzoxy Phe–Met); Sigma, St. Louis, Missouri, fusidic acid, phenylbutazone, sulfinpyrazone, sulfasalazine, sulfapyridine, Hepes buffer; Gibco Laboratories, Grand Island, New York, calcium- and magnesium-free Hanks' balanced salt solution (HBSS) and medium 199; MCI Biomedical, Rockland, Maine, Seakem agarose.

Human polymorphonuclear neutrophils (PMNs) were purified as described previously (20) and were resuspended to 1×10^7/ml in calcium- and magnesium-free Hanks' balanced salt solution containing 0.1% gelatin and buffered to pH 7.2 with Hepes (HHG).

The under-agarose assay of Nelson *et al.* (22) was used with the following modifications. The agarose was 0.8%, buffered to pH 7.2 with Hepes buffer, 5 mg/ml, and contained 0.1% gelatin and medium 199. Wells were punched, 0.25 cm in diameter, three in a row, separated by 0.25 cm. All additions to wells were made using 10-μl vol. PMNs, 1×10^5, were added to the middle well and FMLP, at the concentrations noted, was added to the outer wells. In the countergradient experiments, putative nonchemotactic ligands were added to the in-

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ner wells and were usually added 1 hr before PMNs and FMLP. Incubation was conducted at 37°C for 3 hr and was terminated by the addition of 10% formaldehyde.

The results represent the average of at least four samples and were expressed as the average micrometers (μm ± 1 SD) locomoted by at least 10 PMNs from the edge of the well. FMLP was incorporated into agarose in radial migration experiments (23–27). The distance measured in these experiments was on the PMN well side distal from the well containing the nonchemotactic ligand. In the countergradient experiments, the distance measured was also on the PMN well side distal to the well containing the nonchemotactic ligand and proximal to the FMLP well (See Fig. 1). In terms of variation, the results with each agent were replicated at least twice. The standard deviations of the quadruplicate samples averaged 10% (cf. Figs. 2 and 4). The standard deviation bars were omitted from some figures for the sake of clarity. The experiments selected for presentation were those in which more than one drug was employed in the same experiment: this allowed for direct drug-to-drug comparison. Thus, Figs. 3 and 4 were constructed from the data in one experiment; Figs. 6, 7, and 8 from another. The major source of variation was due to differing donor PMN sensitivity to FMLP concentrations with maximum responses seen at FMLP concentrations over a threefold range.

Results. An example of the preliminary experiments described in the introduction is shown in Fig. 2. In these experiments, FMLP was incorporated into the agarose, the putative nonchemotactic ligands were placed in one well, and the PMNs added to the other. As expected, decreased radial migration accompanied increasing FMLP concentrations. However, when rifampin or fusidic acid was

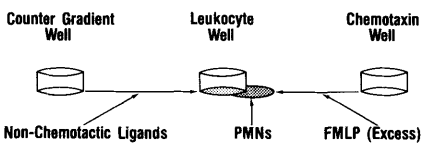

FIG. 1. Countergradient chemotaxis under agarose. Diagram of the system for measuring stimulation of FMLP-induced chemotaxis using countergradients of nonchemotactic ligands for FMLP receptors.

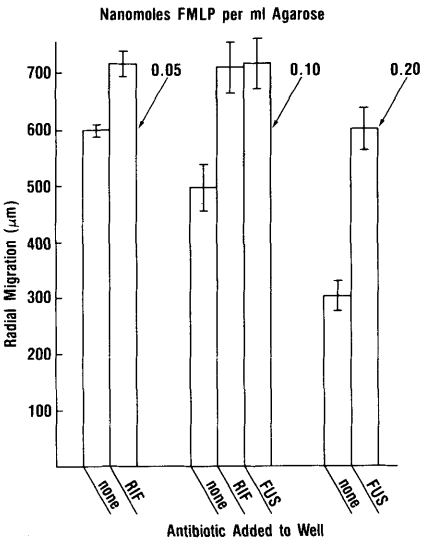

FIG. 2. Effects of rifampin and fusidic acid gradients on FMLP-induced radial migration under agarose. FMLP was incorporated into agarose at the concentrations shown. Rifampin (12 nmole) and fusidic acid (19 nmole) were added to the wells 1 hr before PMNs, followed by 3-hr incubation at 37°C.

added to wells, both compounds reversed the immobilization on the distal side of the PMN well, i.e., on the side opposite from the antibiotic well (inhibition of migration was observed on the side proximal to the antibiotic well). A range of antibiotic concentrations, time of additions, and FMLP concentrations was employed in different experiments. The results in Fig. 2 were obtained with 12 nmole rifampin per well and 19 nmole fusidic acid per well added 1 hr before the addition of PMNs. Using 0.1 nmole FMLP/ml agarose, both rifampin and fusidic acid increased migration (away from their gradients) from 500 to 700 μm. At the next highest FMLP concentration, 0.2 nmole/ml agarose, fusidic acid doubled the distance migrated from 285 to 590 μm.

Although results similar to those described above were obtained in four experiments, no effect was observed in two others. Attempts to improve the reproducibility of the two-well system by changing concentrations, addition times, and manipulation of other experimental parameters were unsuccessful. The reason for the inconsistent results was thought to be related to the difficulty in achieving the necessary

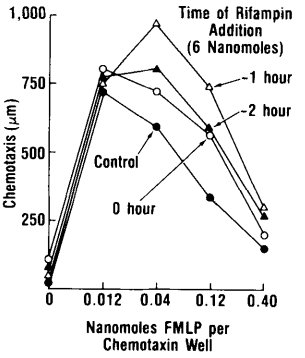

FIG. 3. Effect of adding rifampin to countergradient wells on FMLP-induced chemotaxis. Rifampin, 6 nmole per countergradient well, was added 2, 1, and 0 hr before PMNs (1×10^5 per middle well) and FMLP (added at the concentrations shown to the chemotaxin well). The system was incubated for 3 hr at 37°C.

balance between FMLP concentrations and both the antibiotic concentration and rate of movement of the antibiotic gradient. Thus, the concentration differential across the FMLP receptors on the PMN surface between the two receptor competitors was small and fleeting in time. The question became, therefore, one of increasing this differential. It was hypothesized that this might be accomplished by placing the PMNs in a well separating two other wells containing FMLP and the antibiotic. In this way, a gradient of chemotaxin ligand and a countergradient of nonchemotaxin ligand would be established and this should increase the probability that a greater differential concentration would be achieved across the PMN surface. The results described below suggest that this was achieved in a reliable fashion and using a variety of agents.

Figure 3 displays the results of adding 6 nmole of rifampin to the countergradient well at different times before PMNs and FMLP. At FMLP concentrations of slight excess (0.04 and 0.12 nmole per well), rifampin markedly reversed the immobilization. In some cases, rifampin more than doubled the distance locomoted when added 1 hr before PMNs and FMLP at 0.12 nmole per chemotaxin well. Comparing the results at 0.012 and 0.4 nmole FMLP per well, which were the lowest and the highest FMLP concentrations tested in this experiment, shows clearly the dynamic nature of the phenomenon of reversing immobilization: little or no effect of rifampin was observed at FMLP concentrations that were either too low or too high.

Fusidic acid reversal of immobilization resembled that of rifampin but the data in Fig. 4 emphasized a different point: compounds such as rifampin and fusidic acid not only reversed FMLP-induced immobilization, they often increased locomotion beyond that attained under optimal FMLP concentrations. For instance, maximum locomotion of 725 μm was seen in this experiment at 0.012 nmol FMLP in the chemotaxin well; lower FMLP concentrations resulted in less chemotaxis (data not shown). However, when 9 nmole fusidic acid was added to countergradient wells, either 1 or 2 hr before PMN and FMLP addition (0.04 nmole FMLP), maximum migration was increased from 725 to 925 and 875 μm, respectively. Thus, the PMNs traveled distances under the joint influence of the antibiotics and a slight excess of FMLP which were beyond those obtained at optimal concentrations of FMLP used alone.

Carbobenzoxy Phe–Met was examined in the system because it was the first compound described that antagonized the activity of FMLP (15). Stimulation of FMLP-induced chemotaxis by addition of carbobenzoxy Phe–Met to countergradient wells resembled that observed with rifampin and fusidic acid (Fig. 5). Carbobenzoxy Phe–Met stimulated chemotaxis at concentrations ranging from 12 to 46 nmole per countergradient well (lower concentrations were without effect).

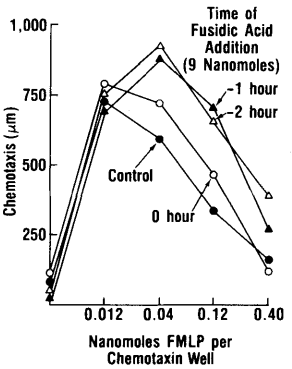

FIG. 4. Effect of adding fusidic acid to countergradient wells on FMLP-induced chemotaxis. The conditions were as described under Fig. 3.

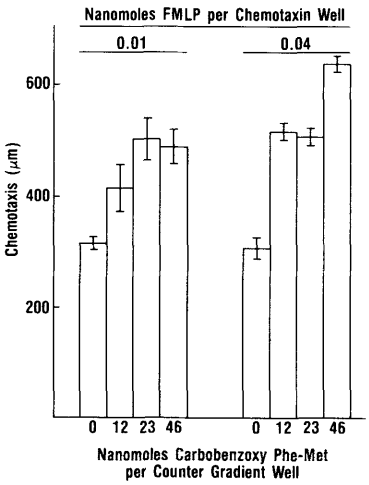

FIG. 5. Effect of adding carbobenzoxy Phe–Met to countergradient wells on FMLP-induced chemotaxis. Carbobenzoxy Phe–Met, at the concentrations shown, was added 1 hr before PMNs and FMLP, followed by 3-hr incubation at 37°C. Concentrations of carbobenzoxy Phe–Met less than 12 nmole per countergradient well had no effect.

Phenylbutazone stimulated chemotaxis across a broad concentration range, from 4 to 32 nmole per countergradient well, depending on the FMLP concentration in the chemotaxin well (Fig. 6). The results with phenylbutazone also confirm (28) and underscore the importance of studying a range of concentrations of nonchemotactic ligands and the chemotaxin: the effects of phenylbutazone ranged from none, to inhibition, to stimulation depending on the conditions employed.

Results with sulfinpyrazone, a pyrazolon derivative related to phenylbutazone (17), are shown in Fig. 7. Similar to phenylbutazone, sulfinpyrazone effects ranged from inhibitory to stimulatory, again emphasizing the concentration interdependence between nonchemotactic ligands and the chemotaxin.

Sulfasalazine and one of its metabolites, sulfapyridine, were studied because of their inhibitory effects on chemotaxis and binding of FMLP (21, 29, 30). As the concentration of sulfasalazine was increased in the countergradient well from 3 to 50 nmole per countergradient well, there was a proportional increase in PMN locomotion induced by 0.12 nmole FMLP per chemotaxin well (Fig. 8). Similar effects were seen at 0.04 nmole FMLP per chemotaxin well. Sulfapyridine significantly increased PMN locomotion only at 40 and 80 nmole sulfapyridine per countergradient well (Fig. 9), and at a lower concentration of FMLP (0.012 nmole per chemotaxin well).

Discussion. The most significant finding using the countergradient system was that a variety of agents considered to be inhibitors of FMLP-induced chemotaxis were, in fact, capable of markedly stimulating locomotion toward FMLP gradients. These agents included carbobenzoxy Phe–Met (15), sulfasalazine and its metabolite, sulfapyridine (21, 29, 30), and fusidic acid. In the case of fusidic acid, its activity in the countergradient system might have been inferred from its ability to inhibit *Escherichia coli* culture filtrate-induced chemotaxis (31, 32) and the finding that FMLP was a major chemotactic component in such culture filtrates (33). But the important point is that several compounds can be added to the two shown previously to possess paradoxical inhibitory/stimulatory activities: rifampin (16, 20) and phenylbutazone (28).

The simplest explanation for the ability of these compounds to stimulate (and inhibit) chemotactic responses toward FMLP was the following. Using three wells in a row, a sharp differential across FMLP receptors on the PMN surface was achieved by placing nonchemotactic ligands in the countergradient well, PMNs in the middle well, and FMLP (in

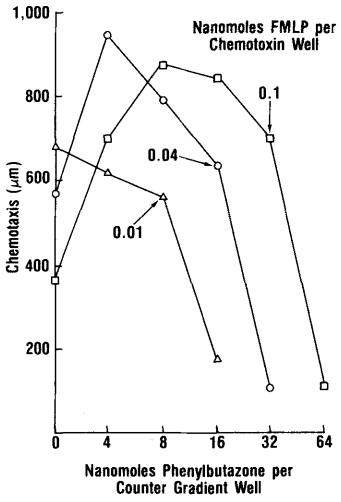

FIG. 6. Effect of adding phenylbutazone to countergradient wells on FMLP-induced chemotaxis. Phenylbutazone, at the concentrations shown, was added 1 hr before PMNs and FMLP, followed by 3-hr incubation at 37°C.

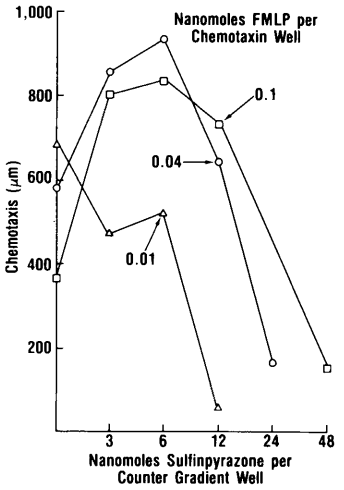

FIG. 7. Effect of adding sulfinpyrazone to countergradient wells on FMLP-induced chemotaxis. Sulfinpyrazone at the concentrations shown, was added 1 hr before PMNs and FMLP, followed by 3-hr incubation at 37°C.

excess) in the chemotaxin well. Thus, the countergradient of competitive ligands displaced FMLP from "near side" receptors while "far side" receptors were still occupied, the leukocyte "sensed" the concentration gradient across its surface, and then moved directionally. In accord with this idea is that many of these agents have been shown to antagonize the binding of radiolabeled FMLP including rifampin (20), phenylbutazone (18, 28), sulfasalazine (21), as well as the first-described FMLP competitive antagonist, carbobenzoxy Phe–Met (15). In addition, a number of corticosteroids decreased the rate of association of FMLP (19) which could explain the stimulatory activity of the steroid antibiotic, fusidic acid.

An alternative explanation to the idea that the active agents stimulated by competing with FMLP at the receptor site is that the agents might be affecting receptor mobilization. A number of investigators have described mobilization of receptors to the leading edge of chemotactically stimulated polarized PMNs (34–37). Thus, slowing down the mobilization could create, in effect, a longer receptor gradient (over time) across the length of the polarized PMN.

In considering the chemistry of the active agents, no obvious structural similarities are apparent. The agents and their chemical class are as follows: rifampin, naphthalenic ansamycin; fusidic acid, steroid; carbobenzoxy Phe–Met, *N*-blocked dipeptide; phenylbutazone and sulfinpyrazone, pyrazolons; sulfasalazine and sulfapyridine, pyridines.

Much data have been acquired concerning the structure activity relationships of FMLP and closely related peptides (1, 2, 38–41), data which led to a model for the FMLP receptor on rabbit PMNs (42). However, a number of unrelated peptides were as potent or efficacious (7, 39). Thus, consideration of the range of the structures of chemotactic agents as well as the inhibitors of FMLP-induced chemotaxis, such as those described in this report, suggest quite broad structural requirements for ligands binding to the FMLP receptor.

In terms of the clinical uses of the agents studied here, rifampin and fusidic acid are antibacterial agents, phenylbutazone is used in arthritic conditions, sulfasalazine to treat ulcerative colitis, and sulfinpyrazone as adjunct therapy in myocardial infarction. Obviously, all these clinical situations involve some aspect of inflammation and, therefore, it is tempting to speculate that FMLP receptors and their functions may play some role in inflammation. However, this speculation must be tempered by the knowledge that many mammals do not respond chemotactically to FMLP including horses (43, 44), pigs (45), cattle (46), dogs (47), and cats (48).

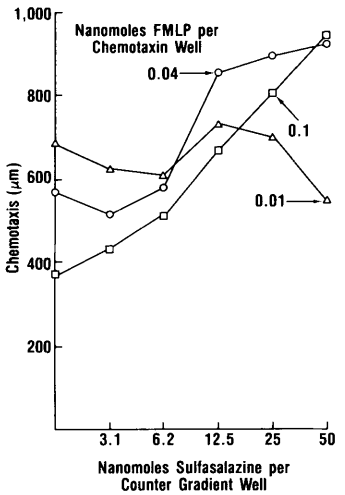

FIG. 8. Effect of adding sulfasalazine to countergradient wells on FMLP-induced chemotaxis. Sulfasalazine, at the concentrations shown, was added 1 hr before PMNs and FMLP, followed by 3-hr incubation at 37°C.

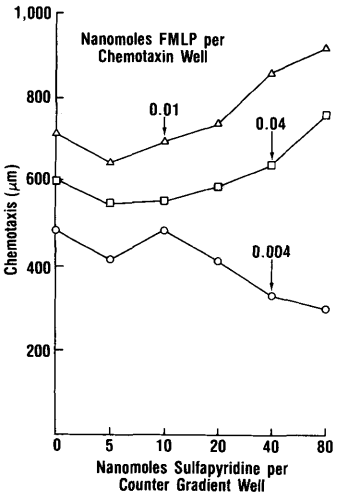

FIG. 9. Effect of adding sulfapyridine to countergradient wells on FMLP-induced chemotaxis. Sulfapyridine, at the concentrations shown, was added 1 hr before PMNs and FMLP, followed by 3-hr incubation at 37°C.

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