ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1983, p. 777–783
0066-4804/83/110777-07$02.00/0

Vol. 24, No. 5

Rifampin Affects Polymorphonuclear Leukocyte Interactions with Bacterial and Synthetic Chemotaxins but not Interactions with Serum-Derived Chemotaxins

GARY D. GRAY,[1]* C. WAYNE SMITH,[2] JAMES C. HOLLERS,[2] DENNIS E. CHENOWETH,[3] VANCE D. FIEGEL,[4] AND ROBERT D. NELSON[4]

Infectious Diseases Research, The Upjohn Company, Kalamazoo, Michigan 49001[1]; Department of Anatomy, Michigan State University, East Lansing, Michigan 48824[2]; Department of Pathology, University of California, San Diego, La Jolla, California 92037[3]; and Department of Surgery, University of Minnesota, Minneapolis, Minnesota 55455[4]

Received 2 June 1983/Accepted 13 September 1983

Three independent experimental approaches support the hypothesis that rifampin competes for receptors on polymorphonuclear leukocytes (PMLs) with small peptide chemoattractants, e.g., *N*-formylmethionylleucylphenylalanine (FMLP), but not with serum-derived chemoattractants (C5a). First, rifampin inhibited chemotaxis induced with FMLP but reversed the immobilization of PMLs that occurred at high FMLP concentrations. Second, rifampin competed with radiolabeled FMLP for binding sites on PMLs and displaced already-bound radiolabeled FMLP. Third, rifampin blocked and reversed the bipolar shape changes induced in PMLs by FMLP. These effects occurred at concentrations attained during rifampin therapy and were not due to rifampin toxicity. In contrast, no effect of rifampin was observed on serum-derived chemoattractants (C5a) in any of the three systems. The evidence suggests, therefore, that rifampin is a ligand for FMLP-type receptors on PMLs.

Chemotaxis of phagocytes toward infectious agents constitutes one important element in successful host defense against infectious disease. Since antibiotics are the agents used most often during therapy of infectious diseases, investigations of the possible influence of antibiotics on chemotaxis are warranted.

In preliminary studies, examination of a panel of antibiotics for their effects on chemotaxis of polymorphonuclear leukocytes (PMLs) confirmed that rifampin was a potent inhibitor of chemotaxis toward *Escherichia coli* culture filtrates (13, 14). However, the unusual shape of dose-response inhibition curves and other data led to the hypothesis that rifampin competes for receptors on PMLs with bacterial and synthetic peptide chemotaxins but not with serum-derived chemotaxins. (G. D. Gray, K. A. Knight, and C. A. Talley, Fed. Proc. 39:878, 1980)

The current study was performed to further evaluate this hypothesis. Three independent experimental approaches were used to study rifampin. We tested its effects on chemotaxis, on the binding of radiolabeled chemotactic ligands to PMLs, and on chemotaxin-induced shape changes in PMLs. Rifampin effects were investigated with two types of chemoattractants that bind to different sites on PMLs: small peptide chemoattractants such as *N*-formylmethionylleucylphenylalanine (FMLP) and serum-derived (C5a) chemoattractants (2, 3, 5, 6, 21, 31).

MATERIALS AND METHODS

Materials. The following reagents were purchased from Vega Biochemicals, Tucson, Ariz.: FMLP, methionylleucylphenylalanine, and carbobenzoxyphenylalanylmethionine (CBZPM); from Sigma Chemical Co., St. Louis, Mo.: *N*-formylmethionylphenylalanine, puromycin, tetracycline, cycloheximide, erythromycin, and pepstatin; from Calbiochem, La Jolla, Calif.: rifampin (rifampicin) and rifamycin SV; from Worthington Biochemicals, Freehold, N.J.: human serum albumin; from MCI Biomedical, Rockland, Mass.: SeaKem agarose; from Aldrich Chemical Co., Milwaukee, Wis.: *N*-methylpiperazine; and from New England Nuclear Corp., Boston, Mass.: *N*-formylmethionylleucyl[^{3}H]phenylalanine (^{3}H-FMLP) (specific activity, 56.9 Ci/mmol). Clindamycin and lincomycin were obtained from the Upjohn Co., Kalamazoo, Mich. Sterile filtrates were prepared from cultures of *Propionibacteria granulosum* (UC-6565) and *E. coli* (UC-51).

Isolation of human PMLs. Two different PML isolation procedures were used. PMLs used in the chemotaxis and radiolabeled-ligand studies were isolated as described previously (5, 21). The PMLs used in the shape change studies were isolated by a slightly different method (9, 30). In both cases, polymorphonuclear neutrophils were present in concentrations of ≥95%. Since eosinophils (1 to 5%) and basophils (0 to 1%)

were sometimes present, the preparations were designated as PMLs.

Chemotaxis assay. The under-agarose chemotaxis assay was conducted similarly to the method described elsewhere (22). The agarose was 0.8% in medium 199, containing 5 mg of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) per ml (pH 7.2), and usually contained 1% human serum albumin. To examine the role that proteins might play in the rifampin effects, the agarose mixture was also prepared with 10% gamma globulin-free horse serum or with no protein. In other experiments, rifampin, FMLP, zymosan-activated serum, or CBZPM was incorporated into the agarose. After addition of the various chemotaxins (identified in the text) to the chemotaxin wells, incubation was usually conducted for 2.5 h at 37°C. The results represent the average of at least three samples and are expressed as the micrometers traveled by at least 10 PMLs from the edge of the well.

^{3}H-FMLP binding assay. The binding of ^{3}H-FMLP to PMLs was conducted as previously described (21). Briefly, ^{3}H-FMLP and PMLs were mixed in tubes in ice to a final concentration of 6×10^{-9} M ^{3}H-FMLP and 1×10^7 PMLs per ml. After 60 min, the PMLs were sedimented by centrifugation at 10,000 × *g* for 30 s, the supernatant was discarded, and PML-associated radioactivity was determined by scintillation spectrometry. Specific binding was defined as the amount of total bound ligand that was displaced by a 1,000-fold excess of unlabeled FMLP present for the duration of the assay. In 20 experiments, specific binding averaged 9,667 ± 389 cpm (mean ± standard error of the mean), whereas nonspecific binding averaged 3,204 ± 156 cpm. To assess the abilities of various compounds to interfere with ^{3}H-FMLP binding, test compounds were added to the reaction mixture either simultaneously with ^{3}H-FMLP or after the introduction of ^{3}H-FMLP. Dimethyl sulfoxide, which was used to dissolve some of the test compounds, had no effect on ^{3}H-FMLP binding at the final concentrations present.

^{125}I-labeled C5a binding assay. C5a was purified to homogeneity by the method of Fernandez and Hugli (12). The glycopeptide was radiolabeled with ^{125}I by the solid-phase lactoperoxidase-glucose oxidase method described by the manufacturer (Technical Bulletin no. 1060; Enzymobead; Bio-Rad Laboratories, Richmond, Calif.). The resulting ^{125}I-labeled C5a was separated from free ^{125}I by immunoaffinity chromatography and had a specific activity of 37 μCi/μg. The binding of ^{125}I-labeled C5a to PMLs was measured as described previously (5). Briefly, 10 μl of ^{125}I-labeled C5a at a concentration of 6×10^{-9} M and 10 μl of the test compound at the concentrations indicated in the text were incubated at room temperature with 80 μl of 3.125×10^6 PMLs per ml of Hanks balanced salt solution containing 0.5% gelatin. After 20 min, the PMLs were sedimented by centrifugation at 10,000 × *g* for 30 s. The amount of ^{125}I-labeled C5a bound to PMLs was calculated from the equation given previously (5).

PML shape change assay. The addition of a chemotaxin causes a change in cellular shape from a spherical to a polarized configuration in a high percentage of PMLs (29, 30). In this study, changes in PML shape were induced by FMLP or C5a. PMLs, 10^6/ml, were incubated with 1 nM FMLP or 26 μg of C5a per ml. The C5a employed in these studies was prepared by a slightly different method than that used in the binding studies described above (15, 30). After incubation at 37°C for various times, the PMLs were fixed at room temperature at a final concentration of 2% glutaraldehyde.

In previous reports, PMLs were classified as round, ruffled, bipolar, or uropod (29, 30). In this report, "percent bipolar" refers to the proportion of PMLs in both the bipolar and uropod categories. Time-lapse cinematography studies confirmed the rapid rate of change observed by using the glutaraldehyde fixative. Repeated direct observation of cells during glutaraldehyde fixation revealed no visible loss of surface configuration after exposure of the PMLs to the fixative.

RESULTS

Rifampin inhibition of chemotaxis toward bacterial culture filtrates. Although rifampin inhibited chemotaxis toward sterile culture filtrates from *P. granulosum*, the potency of this inhibition diminished as the concentration of the chemotactic filtrate was increased (Fig. 1). For instance, at 3 μg of rifampin per ml, the inhibition of chemotaxis, calculated after subtracting the random migration distance (245 μm), was as follows: 84% at 1:8 culture filtrate dilution, 62% at 1:4 dilution, 39% at 1:2 dilution, and only 9%

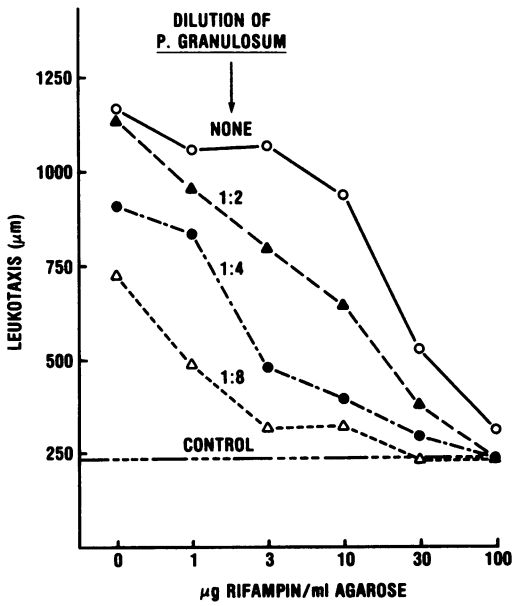

FIG. 1. Rifampin inhibition of chemotaxis toward a *P. granulosum* culture filtrate. A sterile filtrate of a culture of *P. granulosum* (UC 6565) was diluted and added to the chemotactic wells at the concentrations shown above. Control wells received medium 199 only. Rifampin was present in the agarose at the concentrations shown on the abscissa. Human PMLs were added to the cell wells and incubated for 2.5 h at 37°C.

with the undiluted filtrate. In addition, increasingly higher concentrations of rifampin were required to achieve the same degree of inhibition when increasingly higher concentrations of the chemotactic filtrate were employed. Similar results were obtained with a culture filtrate from *E. coli*.

Rifampin inhibited FMLP-induced chemotaxis yet reversed the immobilization that accompanies high FMLP concentrations. FMLP, a chemotactic agent more potent than the bacterial culture filtrates (28), was selected to examine further the apparent interdependence between the chemotaxin and rifampin concentrations. In the absence of rifampin, low (0.1 μg of FMLP per ml) and intermediate (1 μg of FMLP per ml) concentrations of FMLP led to proportionately greater increases in the distances migrated by PMLs (see ordinate, Fig. 2.). However, higher concentrations of FMLP (10 μg/ml) did not result in further increased chemotaxis, confirming the work of others (24, 28). This phenomenon is referred to as "partial immobilization" in this report.

Rifampin inhibited chemotaxis in a dose-response fashion at FMLP concentrations of 0.1 and 1 μg/ml in much the same manner as had been seen with the bacterial culture filtrates.

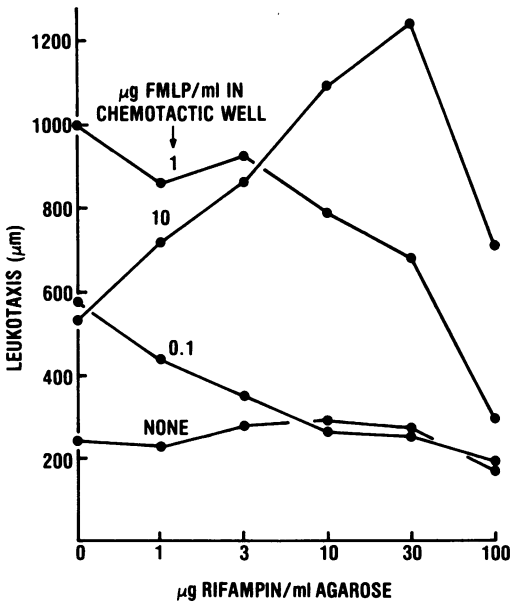

FIG. 2. Rifampin effects on chemotaxis toward FMLP. FMLP was added to chemotactic wells at the concentrations shown above. Control wells received medium 199 only. Rifampin was present in the agarose at the concentrations shown on the abscissa. Human PMLs were added to the cell wells and incubated for 2.5 h at 37°C.

However, rifampin not only failed to further inhibit the partial immobilization observed at 10 μg of FMLP per ml, but it actually reversed the FMLP-induced partial immobilization in a dose-response fashion up to 30 μg of rifampin per ml. Similar results were obtained with rifamycin SV.

To examine the role protein might play in the paradoxical effects of rifampin, the agarose-containing rifampin was prepared with 1% human serum albumin (as usual), 10% gamma globulin-free horse serum, or no protein. Although the total distances migrated by PMLs were less with no protein than with albumin or serum, the results were qualitatively the same in all three situations.

Effects of other antibiotics of FMLP-induced chemotaxis. Tetracycline, erythromycin, clindamycin, and puromycin, which have been reported to be inhibitors of chemotaxis (4, 10, 17–20) were compared with rifampin. The compounds were tested at concentrations ranging from 0.01 to 100 μg/ml of agarose; FMLP was used in concentrations ranging from 0.03 to 10 μg/ml. Tetracycline and erythromycin had no effect on FMLP-induced chemotaxis at any concentration. At 100 μg/ml, clindamycin and puromycin inhibited chemotaxis by an average of about 20% at all FMLP concentrations. Reversal of immobilization was not observed with any of the antibiotics. In the same experiments, rifampin displayed the usual inhibition of chemotaxis at low FMLP concentrations and reversal of immobilization at high FMLP concentrations.

Rifampin effects observed when chemotaxis was induced by other chemotactic peptides. Rifampin was tested for effects on three other chemotactic peptides to assess the generalizability of the effects observed with FMLP. The nonformylated tripeptide methionylleucylphenylalanine, the dipeptide *N*-formylmethionylphenylalanine, and the nonmethionine-containing pentapeptide pepstatin (1) were compared with FMLP. Rifampin had similar influences on the chemotactic responses to all of the chemotaxins, i.e., marked inhibition at lower chemotaxin concentrations but reversal of immobilization at excess chemotaxin concentrations.

Rifampin did not inhibit chemotaxis induced by serum-derived chemotaxins. In contradistinction to the results just described, rifampin had no effect on chemotaxis induced by zymosan-activated serum. At concentrations up to 100 μg of rifampin per ml, no effect was seen on the chemotactic response to either heterologous or autologous zymosan-activated serum. In addition, no rifampin effect was seen on the chemotactic response to heterologous or autologous serum which had been activated by agarose alone (27). These results imply that rifampin had no effect on C5a, since the latter is a major

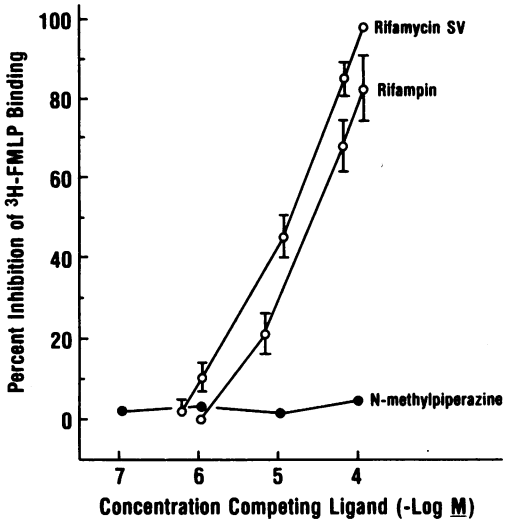

FIG. 3. Influence of various compounds on binding of ^{3}H-FMLP to human PMLs. PMLs were incubated with 6 nM ^{3}H-FMLP in the presence of rifampin, rifamycin SV, or *N*-methylpiperazine at the final concentrations shown above. Data points represent the mean and standard error values derived from duplicate determinations in four experiments.

chemotactic peptide generated by serum activation (5, 12).

Comparison of rifampin effects with those of CBZPM on FMLP-induced chemotaxis. The peptide CBZPM has been shown to antagonize the chemotactic activity of FMLP but not that of C5a (25). Indeed, a family of curves very similar to those shown in Fig. 2 were generated with CBZPM incorporated into the agarose; however, molar concentrations of CBZPM ca. 30 times greater than those of rifampin were required to inhibit FMLP-induced chemotaxis at low FMLP concentrations and to reverse the immobilization seen at high FMLP concentrations (data not shown).

Effects of rifampin on ^{3}H-FMLP binding to PMLs. Rifampin, rifamycin SV, and *N*-methylpiperazine were studied for their effects on ^{3}H-FMLP binding to PMLs (Fig. 3). The latter two compounds were tested because rifampin structurally resembles a coupling of these compounds. The concentrations required to inhibit binding by 50% were 2.5×10^{-5} M for rifampin and 1.3×10^{-5} M for rifamycin SV; *N*-methylpiperazine did not affect specific binding of ^{3}H-FMLP when present at concentrations over the range of 10^{-7} to 10^{-4} M.

Two other antibiotics, lincomycin and clindamycin, were studied for their effects on ^{3}H-FMLP binding because they, like rifampin, have been reported to bind to PMLs (17, 26). However, at concentrations from 10^{-7} to 10^{-4} M, no effects were observed with either compound.

To examine the possibility that rifampin may noncompetitively modify FMLP receptor function, perhaps by a toxic mechanism, the influence of preexposure of rifampin on binding of ^{3}H-FMLP was assessed. PMLs exposed to 10^{-4} M rifampin either in ice or at 37°C for 20 min, washed by centrifugation, and mixed with ^{3}H-FMLP bound the same amount of ^{3}H-FMLP as did the controls. The preceding experiments suggest that rifampin directly and competitively inhibited the binding of FMLP. Such direct competition for a specific receptor would also require that rifampin displace already-bound labeled ligand. To test this effect, the binding of ^{3}H-FMLP was followed at intervals over a 60-min time period (Fig. 4). Rifampin or unlabeled FMLP was added separately to the ^{3}H-FMLP–PML mixture 30 min after the addition of ^{3}H-FMLP, the time at which total binding of the labeled ligand reached an equilibrium under the conditions described. Bound ^{3}H-FMLP decreased by 27 and 40% in the presence of rifampin and unlabeled FMLP, respectively.

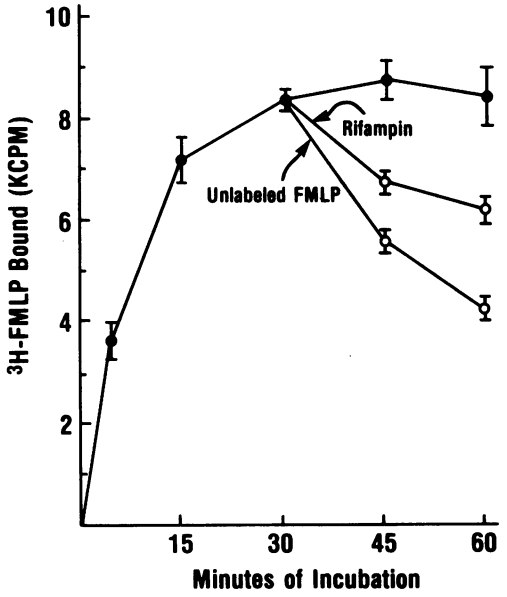

FIG. 4. Displacement of bound ^{3}H-FMLP by rifampin and unlabeled FMLP. PMLs were incubated with ^{3}H-FMLP at a final concentration of 6 nM, and total binding was assessed at intervals over a 60-min period. Thirty minutes after the addition of ^{3}H-FMLP, rifampin or unlabeled FMLP was added to achieve final concentrations of 1.2×10^{-4} and 6×10^{-6} M, respectively. Total binding was assessed over the succeeding 30-min period. Data points represent the mean and standard error values of duplicate determinations in a single experiment.

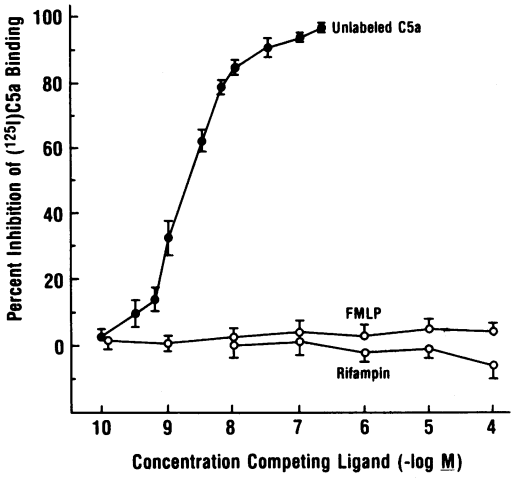

FIG. 5. Influence of rifampin, FMLP, and C5a on the binding of ^{125}I-labeled C5a to human PMLs. PMLs were incubated with 6 nM ^{125}I-labeled C5a in the presence of unlabeled C5a, FMLP, or rifampin at the final concentrations shown above. Data points represent the mean and standard error values of triplicate determinations in a single experiment.

To assess whether rifampin-FMLP complexes are formed and how they might alter the binding properties of the labeled ligand, the absorption spectra of the individual compounds were compared before and after mixing the solutions together for 45 min at 37°C. No change in the absorption spectra was observed, suggesting that the formation of a rifampin-FMLP complex was unlikely.

Effect of rifampin on binding of ^{125}I-labeled C5a to PMLs. Rifampin was tested for its effects on C5a binding since the latter binds to receptors different from those to which FMLP binds (2, 3, 5, 6, 21, 31). In contrast to the inhibitory effects of rifampin on ^{3}H-FMLP, rifampin did not influence the binding of ^{125}I-labeled C5a to its membrane receptor (Fig. 5).

Effects of rifampin on PML shape changes induced by FMLP or C5a. When PMLs were incubated with FMLP or C5a, greater than 85% were converted from a spherical to an elongated bipolar shape (see ordinate, Fig. 6). When rifampin was simultaneously present at the concentrations shown, a dose-dependent inhibition of FMLP-induced shape change was observed. Rifampin had no effect, however, when the shape change was induced by C5a.

Effects of rifampin when added after FMLP-induced PML shape changes. The shape changes induced in PMLs by chemotaxins occur quite rapidly. For instance, when FMLP was added to PMLs, the peak response was observed at 1 to 2 min, followed by a slight downward drift by 4 min (Fig. 7, arrow A). In contrast, when rifampin was added after 2 min, there was a fast reversal to the spherical shape within 0.5 min (Fig. 7, arrow B). When C5a was then added to this preparation (2 min after rifampin had been added), the PMLs readily converted back to the bipolar form even though these PMLs had been previously stimulated by FMLP and then inhibited by rifampin (Fig. 7, arrow C). It should be noted that the response to C5a in this experiment was indistinguishable from previously seen responses of PMLs exposed to C5a only.

When the PMLs were treated with FMLP and then rifampin, washed twice in Hanks balanced salt solution, and re-treated with FMLP, the PMLs responded rapidly to FMLP and became bipolar again (Fig. 7, arrow D).

DISCUSSION

Data from three independent experimental approaches support the hypothesis that rifampin selectively binds to FMLP-type receptors on human PMLs: rifampin inhibited FMLP-induced chemotaxis, ^{3}H-FMLP binding, and shape changes in PMLs induced by FMLP. Rifampin also reversed FMLP-induced immobilization of PMLs and shape changes in PMLs and caused dissociation of ^{3}H-FMLP from PMLs. These findings indicate that rifampin is able to both prevent FMLP binding to receptors on PMLs

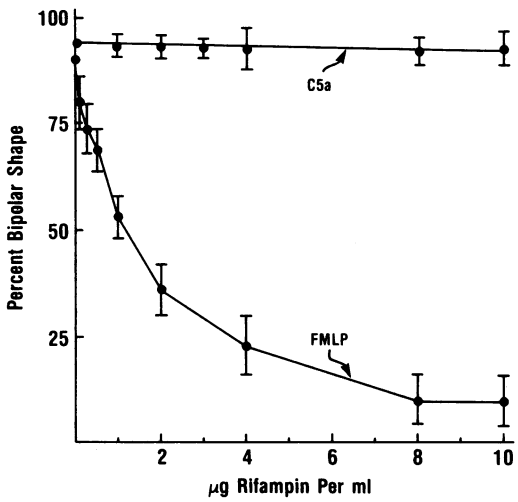

FIG. 6. Rifampin inhibition of FMLP-induced, but not C5a-induced, shape changes in PMLs. PMLs were added to vials containing rifampin at the concentrations shown above and either FMLP (1 nM) or C5a (26 μg/ml). The mixtures were incubated for 10 min at 37°C, and PMLs were fixed in glutaraldehyde. In the absence of rifampin (see ordinate), FMLP and C5a converted over 90% of the PMLs from a spherical to an elongated bipolar shape. The data points represent the mean values from at least three experiments.

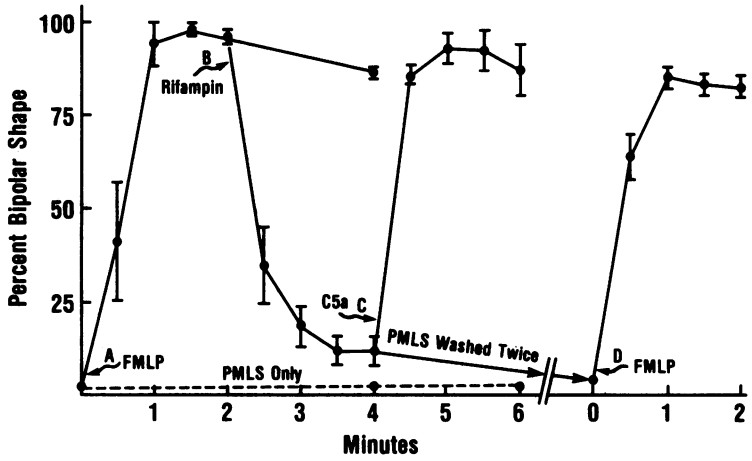

FIG. 7. Rifampin reversal of FMLP-induced shape changes could be overcome by adding C5a or by washing the PMLs. PMLs were added to vials (arrow A) containing FMLP (1 nM). After 2 min, rifampin (10 μg/ml) was added to some vials (arrow B). These vials were then treated one of two ways. C5a (26 μg/ml) was added to one group 2 min later (arrow C). The second group was washed twice by centrifugation in Hanks balanced salt solution and reintroduced to FMLP-containing vials (arrow D). PMLs were fixed in glutaraldehyde at the times shown above.

and displace FMLP that is already bound.

The effects of rifampin appear to be quite specific for the FMLP receptor since rifampin did not affect serum-induced (C5a) chemotaxis, the binding of ^{125}I-labeled C5a to PMLs, or C5a-induced shape changes in PMLs. Since the C5a receptor is known to be different than the FMLP-type receptor (2, 3, 5, 6, 21, 31), rifampin appears to selectively antagonize the binding of ligands for the latter receptors.

The effects of rifampin in these experiments did not occur by a toxic mechanism. First, the effects of rifampin were reversible. Washing rifampin-treated PMLs restored their ability to bind ^{3}H-FMLP and to respond to FMLP by changing to a bipolar configuration. Second, rifampin showed no effect on chemotaxis induced by activated serum (C5a) or on C5a-induced shape changes, both of which require PML viability. Finally, the effects of rifampin reported here were observed at concentrations readily attained in serum during rifampin therapy (7, 8, 11, 16, 23).

Although the results suggest that rifampin and FMLP compete for the same receptor, other mechanisms also could explain these findings. For instance, inhibition of FMLP binding could occur directly or indirectly through (i) insertion of the antibiotic into membrane lipid, (ii) physical modification of receptor structure, (iii) stimulation of the cell to modify receptor function, or (iv) formation of a rifampin-peptide complex. The results presented would seem to exclude several of these alternative mechanisms as explanations for rifampin-mediated inhibition of FMLP binding. Thus, both the inhibition of binding and ^{3}H-FMLP displacement occurred at ice temperature, and this would be expected to prevent or at least minimize solubility of the antibiotic in membrane lipid, antibiotic-mediated rearrangements of receptor structure, or stimulation of the cell to modulate peptide receptor function. Furthermore, if rifampin were acting by one of these mechanisms, it would be expected that preexposure of the PMLs to antibiotic would inhibit binding of the labeled FMLP to some degree. Such pretreatment of the cells, either at ice temperature or at 37°C, followed by washing of the cells, did not change the level of binding of the labeled ligand. Therefore, rifampin and FMLP must both be present for inhibition of binding to occur. Alternative (iv) seems unlikely since no change in absorption spectra was observed when the two compounds were mixed together. Indirect evidence for the idea that rifampin and FMLP compete was obtained also with CBZPM, an antagonist of FMLP (25), which showed inhibitory-stimulatory activities qualitatively the same as those of rifampin.

The idea that rifampin and FMLP compete for the same receptor explains some of the earlier paradoxical findings that rifampin readily inhibited synthetic (and bacterial) chemotaxins when they were employed at low to optimum chemotactic concentrations but that rifampin reversed the immobilization of PMLs that normally occurred at high chemotaxin concentrations (G. D. Gray, K. A. Knight, and C. A. Talley, Fed. Proc. 39:878, 1980). This paradoxical inhibitory-stimulatory action of rifampin also occurred with three other chemotactic peptides known to interact with the FMLP receptor, viz., *N*-for-

mylmethionylphenylalanine, methionylleucylphenylalanine, and pepstatin (21).

Although the effects of rifampin could be reproduced with rifamycin SV, five other antibiotics examined had no activity. These include antibiotics that have been reported to either bind to PMLs or inhibit their chemotaxis, viz., tetracycline, erythromycin, puromycin, clindamycin, and lincomycin (4, 10, 17–20, 26). Thus, rifampin effects appear unusual, they are not shared by other antibiotics that bind to or affect chemotaxis of PMLs, and they seem best explained by specific effects on the FMLP-receptor and its functions.

ACKNOWLEDGMENTS

Work contributed by C.W.S. and J.C.H. was supported by the American Heart Association. Work contributed by D.E.C. was supported by Public Health Service grant AI 18731 from the National Institutes of Health and by grant-in-aid no. 79-863 and an Established Investigatorship from the American Heart Association. Work contributed by R.D.N. and V.D.F. was supported by Public Health Service grant CA23707 from the National Cancer Institute and Research Career Development Award AI 00212 from the National Institutes of Health.

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