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# SULFASALAZINE INHIBITION OF BINDING OF *N*-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE (FMLP) TO ITS RECEPTOR ON HUMAN NEUTROPHILS

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**Abstract**—Sulfasalazine, a drug useful in the therapy of inflammatory bowel disease, was found to block *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced arthritis in rabbits as well as FMLP-induced superoxide production and chemotaxis in human neutrophils *in vitro*. Sulfasalazine was also found to block FMLP binding to human neutrophils with an $I_{50}$ of 10 $\mu$M. The dose–response curve for the inhibition of binding was very similar to the dose–response curves for the inhibition of FMLP-induced neutrophil activation.

The synthetic chemotactic factor, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), binds to a receptor on human neutrophils, monocytes and macrophages [1–3]. FMLP is thought to be an analog of the N-blocked chemotactic peptides synthesized by bacteria [1]. Binding of FMLP to its receptor results in lysosomal enzyme release and activation of the respiratory burst, in addition to the chemotactic effects [4].

Sulfasalazine is an antiinflammatory drug useful in the therapy of inflammatory bowel disease [5]. Although this drug has been used for over 40 years, its mechanism of action is unknown. Recently, we reported that sulfasalazine inhibits the lipoxygenase pathway in stimulated human neutrophils *in vitro* and thus blocks the synthesis of leukotriene $B_4$, a potent chemotactic agent [6]. In an attempt to extend this observation to an *in vivo* model, we studied the effects of sulfasalazine on FMLP-induced arthritis in rabbits [7]. As we report here, sulfasalazine markedly inhibited the FMLP-induced neutrophil accumulation in rabbit knees. Before ascribing the inhibition of FMLP-induced arthritis by sulfasalazine to inhibition of neutrophil lipoxygenase, we studied the effects of sulfasalazine on FMLP binding to neutrophils. These studies were prompted by the observations that some other antiinflammatory drugs inhibit FMLP binding. Among the drugs found to inhibit FMLP binding are indomethacin and sulindac [8], phenylbutazone and sulfinpyrazone [9], and eicosatetraynoic acid, an acetylenic analog of arachidonic acid that blocks the lipoxygenase pathway in neutrophils [10]. In this study we report that sulfasalazine inhibited FMLP binding to neutrophils.

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## METHODS

*Chemotactic factor.* Unlabeled FMLP was purchased from the Sigma Chemical Co., St. Louis, MO, while [$^3$H]FMLP (sp. act. 46.4 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA.

*Medium.* The medium used in this study was Tris (pH 7.4) (50 mM), sodium chloride (100 mM), and calcium chloride (1 mM).

*Preparation of neutrophils.* Human neutrophils were isolated from blood of healthy donors by sequential dextran sedimentation and Ficoll–Hypaque gradient centrifugation [11]. Contaminating erythrocytes were lysed by treatment with 0.85% ammonium chloride for 10 min at 37°. The neutrophils were washed three times and then resuspended in the medium. The purity of neutrophil suspensions averaged 97% as judged by Wright's stain, and viability averaged 98% by eosin Y exclusion.

[$^3$H]*FMLP binding studies.* In the binding assay, neutrophils ($10^7$) were incubated at 0° or 37° with [$^3$H]FMLP (final concentration 40 nM) to measure the total binding or [$^3$H]FMLP plus unlabeled FMLP (final concentration 40 $\mu$M) to measure non-specific binding in a total volume of 0.5 ml of medium in 1.5-ml microfuge tubes [12]. In those conditions in which inhibitors were used, the inhibitor was added to the incubation at the same time as the [$^3$H]FMLP. When the binding reaction was at steady state (120 min at 0° and 30 min at 37°), 250-$\mu$l aliquots were carefully layered over 500 $\mu$l of silicone oil (Versilub F50, General Electric, Waterford, NY) in microfuge tubes. The cells were pelleted by a 30-sec spin in a Microfuge (Beckman Instruments, Palo Alto, CA). After pelleting, the aqueous and oil phases were aspirated, and the tip of the tube was cut-off into a scintillation vial. The cell-associated [$^3$H]FMLP was then counted in a Beckman LS 7000 liquid scintillation counter after the addition of 10 ml Aquasol II counting mixture (New England Nuclear

Corp.). The specific binding was calculated by the difference between total and non-specific binding. The non-specific binding was approximately 25% of the total binding. For Scatchard analysis the cells were incubated with various concentrations of [$^3$H]FMLP (2 nM to 0.5 $\mu$M), and the binding was performed as described above. The Scatchard data were analyzed by a computer program where the overall dissociation constants were calculated from the association kinetics [12–15].

*Assay of superoxide radical generation.* The measurement was made by the reduction of ferricytochrome *c* (horse heart, type II: Sigma Chemical Co.) as previously described [16]. All experiments were performed in duplicate in a volume of 0.3 ml in 1.5-ml size microfuge tubes. Serially added were 75 $\mu$l of medium containing 1 mg/ml glucose and 1 mg/ml bovine serum albumin (BSA), pH 7.4, 75 $\mu$l of cells ($30 \times 10^6$/ml), 75 $\mu$l of ferricytochrome *c* solution (4 mg/ml) and 75 $\mu$l of chemotactic peptide dilution. In some experiments, appropriate concentrations of sulfasalazine were added in the reaction mixture. Tubes were then placed in a 37° water bath and periodically agitated. At indicated times the tubes were removed, and the reaction was stopped by placing the tubes in an ice–water bath. The tubes were centrifuged at 8000 *g* for 1 min in a microfuge, and the supernatant fractions were decanted.

The amount of reduced cytochrome *c* was assayed as follows. A 0.3-ml portion of supernatant fraction was added to 2.8 ml of 0.1 M potassium phosphate buffer, pH 7.4, and the absorbance spectrum was measured at 550 nm in a Gilford spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, OH). With the use of potassium ferricyanide (Fisher Scientific Co., Pittsburgh, PA) and sodium dithionite (J. T. Baker Chemical Co., Phillipsburg, NJ), the amount of reduced cytochrome *c* and the total amount of cytochrome *c* present were calculated with an extinction coefficient of 21.1 $mM^{-1}\,cm^{-1}$ at 550 nm (reduced-oxidized). Medium alone served as control. The addition of 10 $\mu$g/ml superoxide dismutase (Sigma Chemical Co.) to the complete reaction mixtures inhibited FMLP-induced cytochrome *c* reduction by greater than 90%, indicating that the reaction was specific for superoxide.

*Chemotaxis assay.* Chemotactic activities were measured by the Boyden chamber technique utilizing $^{51}$Cr-labeled cells [17–19]. The $^{51}$Cr-labeled cells were placed together with the desired concentration of sulfasalazine in the upper chamber and the chemoattractant FMLP was placed in the lower chamber; results are expressed as chemotactic indices which represent the net percentage of total radioactivity present in the lower filter (cells migrating towards chemotactic factor) less control (net percentage of total radioactivity in lower filter where cells are migrating towards medium).

*FMLP-induced arthritis.* Eight female albino rabbits (3 to 3.5 kg) were injected in one knee with 0.3 ml of saline containing 10 nM FMLP and in the opposite knee with 0.3 ml saline containing 10 nM FMLP and 5 mM sulfasalazine [7]. An injection of saline along into a rabbit knee results in an accumulation of not greater than 500 white blood cells in the synovial fluid. Ninety minutes after injection the animals were killed, the joints were opened, and fluid was collected from the joint with a heparinized micropipette. The number of white blood cells in the joint fluid was counted with the aid of a Coulter counter (Coulter Electronics, Hialeah, FL) [7].

*Chromatography.* [$^3$H]FMLP (100 nM) was incubated with and without sulfasalazine (0.5 mM) in 0.5 ml of phosphate-buffered saline, pH 7.4. An aliquot (0.15 ml) of this mixture was placed on a Bio-Gel P-2 column and eluted with phosphate-buffered saline [10]. Fractions were collected and counted in a liquid scintillation counter.

*Statistical analysis.* The statistical significance was evaluated by Student's paired *t*-test.

## RESULTS

Synovial fluid collected from rabbit knees injected with 10 nM FMLP yielded an average 205,000 white cells/joint of which 98% were neutrophils (Table 1). When sulfasalazine (5 mM) was injected with the FMLP, there was an 81% decrease in the accumulation of neutrophils. To determine if sulfasalazine inhibited other FMLP-induced neutrophil responses, the effects of sulfasalazine on FMLP-induced superoxide production and chemotaxis were studied *in vitro* using human neutrophils. Sulfasalazine (0.1 mM) blocked superoxide production by 58%, and 1.0 mM sulfasalazine totally blocked superoxide production (Table 2). Sulfasalazine (0.1 mM and 1.0 mM) also inhibited FMLP-induced chemotaxis *in vitro* (Table 3).

Binding studies were done to determine if the inhibition of FMLP-induced *in vivo* neutrophil accumulation and *in vitro* chemotaxis and superoxide production by sulfasalazine were related to inhibition of FMLP binding. In the absence of sulfasalazine, 21 fmoles of [$^3$H]FMLP at 0° and 56 fmoles of

Table 1. Effect of sulfasalazine on arthritis induced by FMLP*

| | WBC in synovial fluid† |
|---|---|
| FMLP (10 nM) | 205,193 ± 43,692 |
| FMLP (10 nM) + sulfasalazine (5 mM) | 37,885 ± 31,960‡ |

* Rabbits (8) were killed 90 min after intraarticular injection of FMLP or FMLP plus sulfasalazine. The knee joints were opened, and the joint fluid was collected with a micropipette. Ninety-eight percent of the cells were neutrophils.

† Total cell count ± S.D. ($N = 3$).

‡ $P < 0.0025$ when compared with FMLP alone.

Table 2. Effect of sulfasalazine on FMLP-induced superoxide production*

| | Cytochrome *c* reduced (nmoles/$10^6$ cells) | % Inhibition |
|---|---|---|
| FMLP | 3.6 ± 0.3 | |
| FMLP + sulfasalazine (0.01 mM) | 3.6 ± 0.3 | |
| FMLP + sulfasalazine (0.1 mM) | 1.5 ± 0.1† | 58.3 |
| FMLP + sulfasalazine (1.0 mM) | <0.1 | 100 |

* Neutrophils ($3 \times 10^6$) were incubated in the presence or absence of sulfasalazine with FMLP (100 nM) and ferricytochrome *c* (75 μM). Superoxide release, expressed as nmoles of cytochrome *c* reduced per $10^6$ neutrophils, was assessed after 5 min at 37°. Results represent the mean ± S.D. of three experiments.

† $P < 0.001$ compared to FMLP alone.

Table 3. Effect of sulfasalazine on chemotactic activity of human neutrophils towards FMLP

| | Chemotactic index | P values (compared) to FMLP alone) |
|---|---|---|
| FMLP (1 nM) | 9.1 ± 1.8* | |
| FMLP (1 nM) + sulfasalazine (0.01 mM) | 6.9 ± 2.0 | NS† |
| FMLP (1 nM) + sulfasalazine (0.1 mM) | 3.8 ± 0.9 | <0.025 |
| FMLP (1 nM) + sulfasalazine (1.0 mM) | 0.4 ± 0.2 | <0.025 |

* Mean ± S.E.M. of four experiments, each performed in triplicate.

† Not significant.

Table 4. Effect of sulfasalazine and 5-aminosalicylate on [$^3$H]FMLP binding*

| | Concn (mM) | [$^3$H]FMLP binding at 37° (fmoles/2.5 × $10^6$ cells) | % Inhibition | [$^3$H]FMLP binding at 0° (fmoles/2.5 × $10^6$ cells) | % Inhibition |
|---|---|---|---|---|---|
| Control | | 56 | | 21 | |
| Sulfasalazine | 0.01 | 27 | 51.5 | 11 | 47.6 |
| | 0.1 | 13 | 76.7 | 7 | 66.7 |
| | 1.0 | 2 | 96.4 | 2 | 90.5 |
| | 2.0 | 0.6 | 98.9 | ND† | |
| | 4.0 | 0.8 | 98.7 | ND | |
| | 5.0 | 0.2 | 99.7 | ND | |
| 5-Aminosalicylate | 1.0 | 45 | 19.6 | 19.2 | 9.5 |
| | 2.0 | 44 | 21.4 | 17.0 | 19.0 |
| | 3.0 | 41 | 26.7 | 16.6 | 20.7 |
| | 4.0 | 37 | 33.9 | 14.9 | 29.0 |
| | 5.0 | 32 | 42.9 | 13.6 | 35.2 |

* Neutrophils ($1 \times 10^7$) were incubated with [$^3$H]FMLP (100 nM) in the presence and absence of inhibitors for 30 min at 37° or 120 minutes at 0°. Data presented are means of three experiments. In each case the S.D. was less than 5% of the mean.

† Not done.

Table 5. Effect of sulfasalazine on the dissociation constants for high ($K_d$H) and low ($K_d$L) affinity FMLP receptors on human neutrophils*

| | 37° | | | | 0° | | | |
|---|---|---|---|---|---|---|---|---|
| | $K_d$H (nm) | | $K_d$L (nm) | | $K_d$H (nm) | | $K_d$L (nm) | |
| | Expt. I | Expt. II | Expt. I | Expt. II | Expt. I | Expt. II | Expt. I | Expt. II |
| Control | 0.16 | 0.18 | 1.1 | 1.3 | 0.18 | 0.21 | 1.2 | 1.5 |
| Sulfasalazine | | | | | | | | |
| 0.01 mM | 0.15 | 0.18 | 1.5 | 2.1 | 0.16 | 0.19 | 1.3 | 1.8 |
| 0.1 mM | 0.10 | 0.13 | 1.3 | 1.8 | 0.12 | 0.14 | 1.2 | 1.5 |
| 1.0 mM | 0.05 | 0.07 | 0.7 | 0.9 | 0.03 | 0.05 | 0.5 | 0.8 |

* Neutrophils were incubated with various concentrations of [$^3$H]FMLP for 30 min at 37° or for 120 min at 0°.

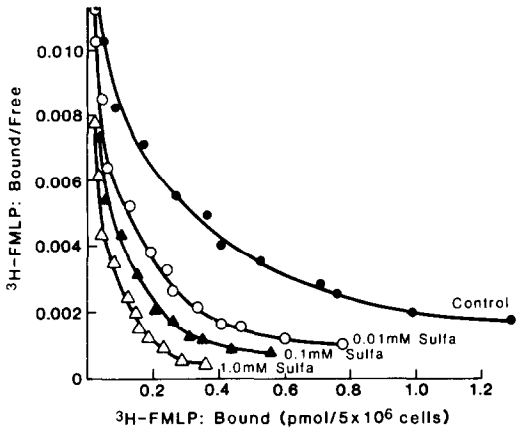


Fig. 1. Scatchard plots of [$^3$H]FMLP binding to human neutrophils at 37° in the presence of various concentrations of sulfasalazine. The binding was performed for 30 min.

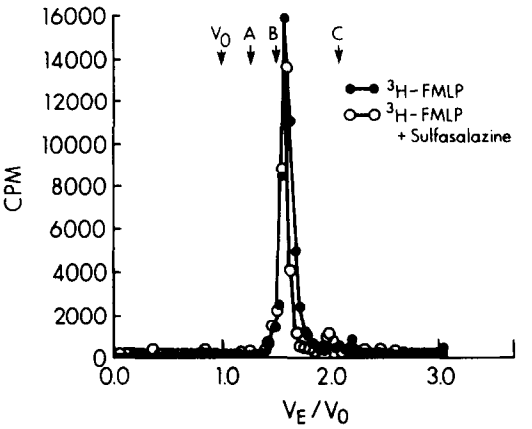


Fig. 3. Bio-Gel P-2 column chromatography. [$^3$H]FMLP ($1 \times 10^{-7}$ M) was incubated either alone or with sulfasalazine (0.5 mM) in phosphate-buffered saline (pH 7.4) (PBS) for 5 min at 37°. Aliquots were loaded in a Bio-Gel P-2 column and eluted with PBS. The eluted fractions were collected and radioactivity was measured in a liquid scintillation counter. The Bio-Gel P-2 column was $1 \times 23$ cm, 18 ml bed volume. Flow rate was 4.4 cm/hr. $V_0$ = 9.4 ml. Key: (A) oxidized glutathione (mol. wt = 612 daltons); (B) reduced glutathione (mol. wt = 307 daltons); and (C) sodium azide (mol. wt = 65 daltons).

[$^3$H]FMLP at 37° were bound by $2.5 \times 10^6$ cells. At 0° greater than 47% of [$^3$H]FMLP binding was inhibited by 0.01 mM sulfasalazine and greater than 90% by 1 mM sulfasalazine (Table 4). Inhibition of FMLP binding by sulfasalazine was similar at 0° and 37°. Although sulfasalazine inhibited specific FMLP binding, it had no effect on non-specific binding (data not shown). The inhibition of FMLP binding, FMLP-induced superoxide production and chemotaxis of neutrophils correlated well. The concentration of sulfasalazine (0.1 mM) that blocked binding 76.7% at 37° and 66.7% at 0° blocked superoxide production by 58.3% and chemotactic response by 58.2%, and the concentration (1.0 mM) that blocked binding by 96% at 37° and 90% at 0° totally blocked superoxide production and blocked chemotactic response by 95.6%. Scatchard analysis performed at 0° as well as 37° revealed (Figs. 1 and 2) that sulfasalazine at concentrations up to 0.1 mM had no effect on the dissociation constants for either the high or low affinity FMLP receptor (Table 5). Sulfasalazine (1 mM) caused a decrease in the dissociation constant for the high affinity receptor. The finding that at the concentrations up to 0.1 mM sulfasalazine blocked FMLP binding without affecting the high or low dissociation constants suggests that sulfasalazine acts as a competitive inhibitor.

The cleavage product of sulfasalazine, 5-aminosalicylate, also affected [$^3$H]FMLP binding at both 0° and 37° but to a much lesser extent than sulfasalazine (Table 4). 5-Aminosalicylate (1 mM) inhibited FMLP binding by 19.6% at 37°, whereas the same concentration of sulfasalazine inhibited FMLP binding by 96.4%.

The inhibition of FMLP binding to its neutrophil receptor by sulfasalazine suggests two possible explanations: either sulfasalazine interacts with FMLP in solution preventing its binding to the receptor or sulfasalazine competes for the receptor. To determine if FMLP and sulfasalazine interact in solution, [$^3$H]FMLP was incubated in the presence and absence of sulfasalazine, and the incubation mixture was loaded on a Bio-Gel P-2 column. FMLP eluted from the column with a $V_e/V_0$ of 1.6 which is close to what would be expected based on its molecular weight (mol. wt = 337 daltons). The radioactivity eluted as a single peak whether sulfasalazine was present or not (Fig. 3), suggesting that FMLP does not complex with sulfasalazine in solution. Sulfasalazine, which is yellow in color, elutes with $V_e/V_0 = 6.60$ (determined colorimetrically) which is greater than one would expect on the basis of its molecular weight and is presumably due to hydrophobic interaction with the gel. When sulfasalazine was incubated with FMLP, all of the yellow color eluted from the column as a single peak with the same $V_e/V_0$ as sulfasalazine alone. Thus, if FMLP and sulfasalazine are incubated together and chromatographed on a Bio-Gel P-2 column, they elute separately, each with the same $V_e/V_0$ as when incubated alone. This suggests that sulfasalazine and

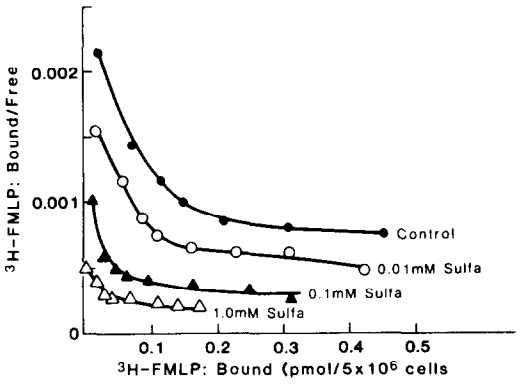


Fig. 2. Scatchard plots of [$^3$H]FMLP binding to human neutrophils at 0° in the presence of various concentrations of sulfasalazine. The binding was performed for 120 min.

FMLP do not form complexes in solution. It is possible that a weak complex is formed in solution that breaks on the column. There is no way to absolutely exclude this possibility. The absence of an effect of sulfasalazine on non-specific FMLP binding also suggests that sulfasalazine and FMLP do not form complexes.

## DISCUSSION

The data presented here demonstrate that sulfasalazine inhibited FMLP binding to human neutrophils. These data suggest that inhibition by sulfasalazine of the FMLP-induced accumulation of neutrophils in the rabbit knee and of FMLP-induced superoxide production and *in vitro* chemotaxis may be secondary to impaired binding of the chemotactic factor to its receptors. Sulfasalazine blocked 50% of FMLP binding at a concentration of 10 $\mu$M. The relatively low concentration of sulfasalazine required to inhibit FMLP binding is of particular interest in view of the pharmacokinetics of sulfasalazine. Sulfasalazine is cleaved in the colon to form 5-aminosalicylate and sulfapyridine. Sulfapyridine is well absorbed but sulfasalazine and 5-aminosalicylate are poorly absorbed. As a result, the concentration of sulfasalazine in the stool is very high (5 mM) while the concentration in the serum is much lower; the average peak concentration after a 2 g oral dose in normal human volunteers has been reported to be 50 $\mu$M [20]. Thus, the $I_{50}$ for inhibition of FMLP binding by sulfasalazine (10 $\mu$M) is a concentration easily obtained in the serum of treated individuals. This is in marked contrast to many of the other described pharmacologic effects of the drug which require concentrations greater than can be achieved in the serum. The concentration of sulfasalazine required for 50% inhibition of prostaglandin $E_1$ ($PGE_1$) accumulation in intestinal mucosa is 250 $\mu$M [21], for 50% inhibition of $PGF_2$ breakdown 50 $\mu$M [22], and for 50% inhibition of neutrophil lipoxygenase 1 mM [6]. The inhibition of FMLP binding by sulfasalazine does not negate any of the previously described pharmacologic effects of the drug; however, in considering the therapeutic effect of sulfasalazine, it is interesting that the inhibition of FMLP binding occurs at a lower concentration of sulfasalazine than the other described pharmacologic effects.

This is the first report of an effect of sulfasalazine on receptor binding and the first report of an effect of sulfasalazine on FMLP-induced neutrophil responses. In an earlier study, Molin and Stendahl [23] reported that sulfasalazine inhibits neutrophil random migration (30% inhibition with 0.1 mM) but not chemotaxis, using normal human serum as an attractant. In that same study, they found that sulfasalazine inhibits phagocytosis, lysosomal enzyme release, and superoxide generated in response to serum-coated zymosan. In each case the inhibition was less than 50% with the highest dose of sulfasalazine tested (0.5 mM), and the mechanism of inhibition was not defined. Certainly, however, the inhibition of particle binding to the cell surface is a possible mechanism. Rhodes *et al.* [24] reported that sulfasalazine inhibits human neutrophil chemotaxis when casein is used as the attractant. The concentration yielding 50% inhibition was 2.5 mM. Whether the inhibition of neutrophil motility in these studies was due to interference with receptor function was not studied. The mechanism of chemotaxis in response to casein is not as well established as with FMLP, and it is not known if a specific surface receptor is involved.

Sulfasalazine is an antiinflammatory drug; its usefulness is best established in the treatment of inflammatory bowel disease. Neither the mechanism of action of sulfasalazine nor the pathogenesis of inflammatory bowel disease is well understood. Despite an extensive search, no infectious etiology has been established for inflammatory bowel disease; however, one current hypothesis for the pathogenesis of inflammatory bowel is an interaction between an infectious agent and a genetically predisposed host. In this study we have demonstrated that sulfasalazine, a drug useful in the therapy of inflammatory bowel disease, blocked the binding of FMLP to its receptor on human neutrophils and, therefore, its biologic response. This receptor is thought to be the receptor for bacteria-derived peptides that activate the inflammatory response to bacterial infections [1]. It is possible that the inhibition of binding of bacterial chemotactic factors to these receptors may account for the efficacy of sulfasalazine in the therapy of inflammatory bowel disease.

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