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EFFECTS OF IBUPROFEN ON CHEMOTACTIC PEPTIDE–RECEPTOR BINDING AND GRANULOCYTE RESPONSE*

KEITH M. SKUBITZ† and DALE E. HAMMERSCHMIDT
Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455, U.S.A.

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Abstract—Inhibition of complement-mediated granulocyte aggregation has been proposed recently as a mechanism of action of high-dose corticosteroids and ibuprofen in shock states. Such inhibition by corticosteroids may be effected through alteration of receptor function, and we have therefore examined the effect of ibuprofen on the extent and kinetics of binding of the synthetic chemotactic peptide formyl-methionine-leucine-phenylalanine (FMLP) to its specific receptor on the granulocyte surface. Dose-dependent inhibition of binding was observed at ibuprofen concentrations paralleling plasma levels achieved with 30 mg/kg intravenous bolus therapy, and also at concentrations achieved with oral therapy. Ibuprofen did not affect the receptor number, but did decrease the association rate constant for the FMLP-receptor interaction (30% of normal for 0.125 mg/ml ibuprofen), leading to a decrease in receptor affinity for ligand. Dissociation kinetics, as determined by cold chase experiments, were unaltered by ibuprofen. We conclude that ibuprofen, like corticosteroids, can slow the rate of association of FMLP with its receptor on the granulocyte surface while allowing dissociation to proceed; altered kinetics of receptor–FMLP interaction may explain the inhibition of granulocyte aggregation. Blockade of granulocyte surface receptors for inflammatory stimuli may be important in the clinical effects of very high-dose corticosteroids and ibuprofen such as are administered in shock; such effects are seen at blood levels of ibuprofen that occur with oral therapy. Similar observations may hold for other physiologic stimuli.

Recent evidence has suggested that complement-stimulated granulocytes are important in the pathogenesis of a variety of clinical disorders [1–10]. High-dose corticosteroids inhibit granulocyte responses to activated complement *in vitro* and *in vivo* [1, 11, 12], and this inhibition might explain in part the reported efficacy of high-dose corticosteroids in some clinical settings [1, 13–22]. We have also shown that high-dose corticosteroids inhibit the binding of the synthetic chemotactic peptide *N*-Formyl-Met-Leu-Phe (FMLP‡) to its specific receptor on the granulocyte surface, and suggested that this inhibition may be a mechanism of the effect of high-dose corticosteroids on granulocyte responses to chemotactic stimuli [23].

Recently, we reported that high-dose corticosteroids and ibuprofen (IB) are synergistic in inhibiting some granulocyte responses to activated complement or FMLP, and additive in inhibiting other responses [24]. Since corticosteroids have been shown to inhibit cell surface receptor function, we examined the effect of IB on the interaction of the granulocyte chemotactic peptide receptor with its ligand. We used the synthetic chemotactic peptide FMLP as a probe to study the effect of IB, alone and in combination with corticosteroids, on granulocyte receptor function. Although its receptor is distinct from that of the chemotactic complement fragment, C5a [25], FMLP mimics many of the effects of activated complement, including chemotaxis and aggregation of granulocytes, and *in vivo* induction of neutropenia [23, 26]. Furthermore, FMLP-induced granulocyte aggregation is inhibited by the same concentrations of IB and corticosteroids as inhibit C5a-induced aggregation [1]. We found that IB, like corticosteroids, inhibited the binding of FMLP to its receptor. This inhibition was due to a decrease in the association rate constant for the FMLP-receptor interaction, leading to a decrease in receptor affinity for ligand. Dissociation kinetics and receptor number were unaltered.

METHODS

Cell preparation. Granulocytes were prepared from heparinized human venous blood by the previously described modification of the method of Boyum [6, 27]. Cells were suspended at appropriate concentrations in Hanks' Balanced Salt Solution (HBSS), pH 7.4 (M. A. Bioproducts, Bethesda, MD); HBSS containing 5 mg/ml bovine serum albumin (HBSS-BSA) (Sigma, St. Louis, MO); or in

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† Author to whom correspondence should be addressed: Keith M. Skubitz, M.D., Box 325, University of Minnesota Hospitals, Minneapolis, MN 55455, U.S.A.

‡ Abbreviations: FMLP, *N*-Formyl-Met-Leu-Phe; IB, ibuprofen; MP, methylprednisolone; HBSS, Hanks' Balanced Salt Solution; BSA, bovine serum albumin; HSA, human serum albumin; DEX, dexamethasone; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; and ZAP, zymosan-activated plasma.

HBSS containing 5 mg/ml human serum albumin (HBSS-HSA) (Cutter Biologicals, Berkeley, CA). Differential cell counts performed on Wright's stained cells revealed greater than 95% PMNs. Cells were greater than 98% viable as determined by trypan blue dye exclusion.

Preparation of solutions. Preservative and filler-free methylprednisolone sodium succinate (MP) and sodium IB (Upjohn Co., Kalamazoo, MI) and dexamethasone sodium phosphate (DEX) (Merck Sharp & Dohme, West Point, PA) were provided by the manufacturers and dissolved in isotonic phosphate-buffered saline (PBS), pH 7.4.

FMLP (Peninsula Laboratories, Inc., San Carlos, CA) was dissolved in dimethyl sulfoxide (DMSO) at 10 mM, diluted in PBS to 1 mM, and stored at −70° in aliquots until use.

Zymosan-activated plasma (ZAP) was prepared as previously described [6] by incubating 2 mg zymosan per ml of heparinized (1 unit/ml) human plasma for 30 min at 37°, followed by removal of zymosan by centrifugation at 10,000 *g* for 30 min at 4°. Aliquots were stored at −70° until use.

Disodium succinate (Sigma) and [^{3}H]FMLP (sp. act. 56.9 Ci/mmole, New England Nuclear, Boston, MA) were dissolved in PBS at appropriate concentrations.

Kinetic analysis of the FMLP receptor interaction. Whereas the relationships between the kinetics of interaction of ligands and cell-bound receptors as compared to the interaction of ligands and solubilized molecules may be quite complex, it is possible to obtain useful information using live cells [28–30]. Analysis of equilibrium binding of FMLP receptors was determined as described by Williams *et al.* [25] with minor modifications [23]. Cells were suspended in PBS containing 0.5 mg/ml BSA (PBS–BSA) at 5–10 × 10^7 cells/ml. One hundred microliters of cell suspension was mixed with 17 μl of corticosteroid, IB, or PBS and incubated for 2 min at 22°; the cells were then cooled to 4° and added to 35 μl of various concentrations of [^{3}H]FMLP and 15 μl of either PBS or 10^{-4} M unlabeled FMLP in a 12 × 75 mm polypropylene tube and incubated at 37° or 4° as indicated for 20 min with occasional shaking. Each sample was then mixed with 3 ml of 0° PBS-BSA, filtered through a Whatman GF/C filter, and the retentate was washed with 12 ml of 0° PBS-BSA. Filters were then dried, added to 6 ml of Aquasol-2 (New England Nuclear, Boston, MA), and counted in a Beckman scintillation counter. Unless otherwise specified, all data are expressed as specific binding (binding of [^{3}H]FMLP in buffer minus binding of [^{3}H]FMLP in the presence of 10^{-5} M unlabeled FMLP). Time-course experiments revealed that specific [^{3}H]FMLP binding to whole cells at 4° plateaued by 20 min. Equilibrium binding data were analyzed by the method of Scatchard [31], where $B/F = B_0K_{eq} - BK_{eq}$, and B = concentration of bound [^{3}H]FMLP, B_0 = total receptor concentration, and F = free [^{3}H]FMLP concentration.

Dissociation kinetics were determined by incubating 5 × 10^7 cells/ml with receptor-saturating concentrations of [^{3}H]FMLP in HBSS at 4° for 20 min. To control for nonspecific binding, parallel experiments were run in which excess unlabeled FMLP was present prior to the addition of the radiolabeled compound. A large excess of unlabeled FMLP or IB (final concentration 5 mg/ml) was then added in a small volume at $t = 0$. At various time intervals, aliquots were taken, filtered through a Whatman GF/C filter, washed with 0° HBSS-BSA, and the filters counted as described above [23]. The concentration of cell-bound [^{3}H]FMLP was analyzed assuming the relationship: $RF \xrightarrow{k_r} R + F$ or $d/dt\,[RF] = -k_r\,[RF]$, where $[RF]$ = the concentration of bound receptor–[^{3}H]FMLP complex, $[R]$ = the free receptor concentration, and $[F]$ = the free [^{3}H]FMLP concentration [23, 32].

Association kinetics were determined by incubating cells in HBSS in the presence or absence of 0.125 mg/ml IB for 2 min at 22°, the cells were then cooled to 4° and [^{3}H]FMLP was added such that the initial [^{3}H]FMLP concentration was at least ten times the initial concentration of receptor sites (determined by equilibrium binding in the presence of excess [^{3}H]FMLP). At various time intervals, samples were removed, filtered, washed, and counted as described above. The correction for nonspecific binding, although performed, was insignificant in most association experiments. The concentrations of bound and free [^{3}H]FMLP were analyzed assuming the relationship:

$$R + F \underset{k_r}{\overset{k_f}{\rightleftharpoons}} RF$$

or $d/dt\,[RF] = k_f\,[R]\,[F] - k_r\,[RF]$, where $[R]$ = the free receptor concentration and $[F]$ = the free [^{3}H]-FMLP concentration. Since $k_r \ll k_f$ in this system, we may ignore the dissociation reaction during the brief time course of these experiments. To confirm that the reaction follows second-order kinetics, several experiments were also analyzed using the general solution for the second-order reaction: $d/dt\,[\ln([R_0]-[RF])/([F_0]-[RF])] = k_f([R_0]-[RF])$, where $[F_0]$ = the initial concentration of [^{3}H]FMLP and $[R_0]$ = the initial receptor concentration [23, 32]. When $[F_0] \gg [R_0]$, we may analyze this reaction as one of pseudo-first-order kinetics, that is: $d/dt\,[RF] = k_f\,[F_0][R]$. Integrating, this becomes: $\ln([R_0]-[RF]) = \ln([R_0]/[F_0]) - k_f[F_0]t$ or $d/dt\,[\ln([R_0] - [RF])] = -k_f[F_0]$ [32].

Granulocyte aggregation. Granulocyte aggregation was performed as previously described [6]. Fifty microliters of ZAP or FMLP, in PBS, was added to 450 μl of a granulocyte suspension (1.1 × 10^7 cells/ml in HBSS-HSA) containing the indicated concentration of IB or MP, which was being stirred at 37° at 900 rpm in a Payton 300B aggregometer/recorder system (Payton Associates, Buffalo, NY). The state of granulocyte aggregation was also confirmed by light microscopy of aliquots taken from the aggregometer.

Reversal of inhibition. Granulocytes (10^7/ml in HBSS-HSA) were exposed to the indicated concentration of MP, IB, or PBS for 2 or 10 min as indicated at 22° and then washed by adding 10 ml of 0° HBSS-BSA, and centrifuging at 400 *g* for 5 min. This wash procedure was repeated as indicated, and the cells were then resuspended to an appropriate volume in HBSS or HBSS-BSA.

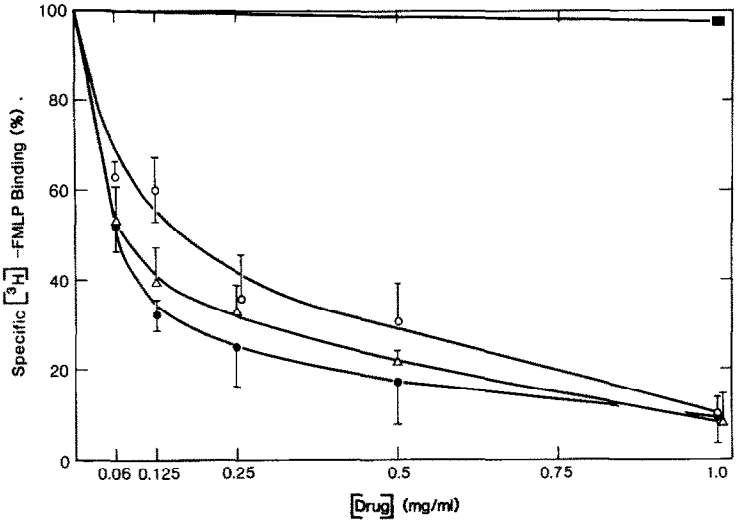

Fig. 1. Effects of ibuprofen and methylprednisolone on FMLP binding to specific surface receptors on live human neutrophils. Specific [^{3}H]FMLP binding at equilibrium in the presence of various concentrations of ibuprofen (○), methylprednisolone (●), a 1:1 by weight mixture of ibuprofen and methylprednisolone (△) (1 mg/ml = 0.5 mg/ml IB + 0.5 mg/ml MP), and sodium succinate (■) at 0° was performed as described in the text. Specific [^{3}H]FMLP binding is expressed as percent binding observed in the absence of drug. The [^{3}H]FMLP concentration was 36 nM. Each point represents the mean ± S.D. of three separate experiments, each of which was performed in triplicate.

RESULTS

Equilibrium FMLP binding. IB and MP both inhibited binding of FMLP to the granulocyte surface, whereas sodium succinate alone had no effect (Fig. 1). Inhibition of binding was dose dependent, and both IB and MP were of similar potency, as was previously observed in aggregation blockade. IB was additive with MP in blocking FMLP binding (Fig. 1). FMLP binding was inhibited by 50% at ~ 0.16 mM MP or ~ 0.62 mM IB. Scatchard analysis of [^{3}H]FMLP binding at various [^{3}H]FMLP concentrations, in the presence and absence of 0.125 mg/ml MP, 0.125 mg/ml IB, and 0.0625 mg/ml IB plus 0.0625 mg/ml MP, revealed no decrease in the number of surface FMLP receptors in the presence of drug (Fig. 2). Both IB and MP as well as a mixture of the two reduced the apparent equilibrium binding constant (Fig. 2). Neither IB nor MP at the concentrations used here altered cell viability, as determined by trypan blue dye exclusion, and viability did not change during the binding assay.

Dissociation kinetics of FMLP binding. The dissociation rate constant for the peptide–receptor interaction, k_r, was determined in the presence of excess unlabeled FMLP and in the presence of 5 mg/ml IB. As previously reported [23, 33], not all bound [^{3}H]FMLP dissociated from the cell (not shown), which probably represents internalization of peptide that is known to occur [33–35]. Much larger fractions of radiolabel remained cell-associated when such experiments were performed at 37° (not shown), as has also been reported [23, 29, 33, 34, 36, 37]. When the initial portion of the dissociation curve was corrected by subtraction of the amount of nondissociating [^{3}H]FMLP, approximately 85% of the total [^{3}H]FMLP bound prior to the addition of unlabeled FMLP dissociated with an apparent k_r of 1×10^{-2} sec^{-1} (Fig. 3). The time–course of receptor–ligand dissociation following the addition of excess unlabeled FMLP was approximately the same as that observed following addition of MP (not shown) or IB (Fig. 3) to a final concentration of 5 mg/ml. The observed k_r (mean of two separate experiments) was 1.2×10^{-2} sec^{-1} in the presence of excess unlabeled FMLP and 0.9×10^{-2} sec^{-1} in the presence of 5 mg/ml IB. Simultaneous addition of 5 mg/ml IB and excess unlabeled FMLP produced similar results (not shown). These values of k_r are comparable to those observed for the structurally similar chemotactic peptide *N*-formyl-norleucyl-leucyl-phenylalanine to its receptor on rabbit neutrophils [33].

Association kinetics of FMLP binding. Analysis of association data as a second-order association reaction as described above yielded a straight line, confirming second-order kinetics (not shown) [23]. To simplify the analysis, further experiments were performed under conditions yielding pseudo-first-order kinetics in the presence of a 10-fold excess concentration of peptide over receptor (Fig. 4) [23]. A single class of forward rate constants is suggested by the linearity of the plot both in the presence and absence of 0.125 mg/ml IB. IB markedly decreased the association rate constant from 2.7×10^5 l/mol-sec to 0.86×10^5 l/mol-sec. Because the observed k_r at 4° was non-trivial, the calculation of accurate k_f values was difficult: association kinetics in the presence and absence of 0.125 mg/ml IB were studied in three experiments, yielding observed k_f values of 2.3 ± 0.4 (S.D.) $\times 10^5$ l/mol-sec and $0.8 \times 10^5 \pm 0.2$ (S.D.) $\times 10^5$ l/mol-sec. These values are comparable to the association rate constants found by others for structurally similar chemotactic

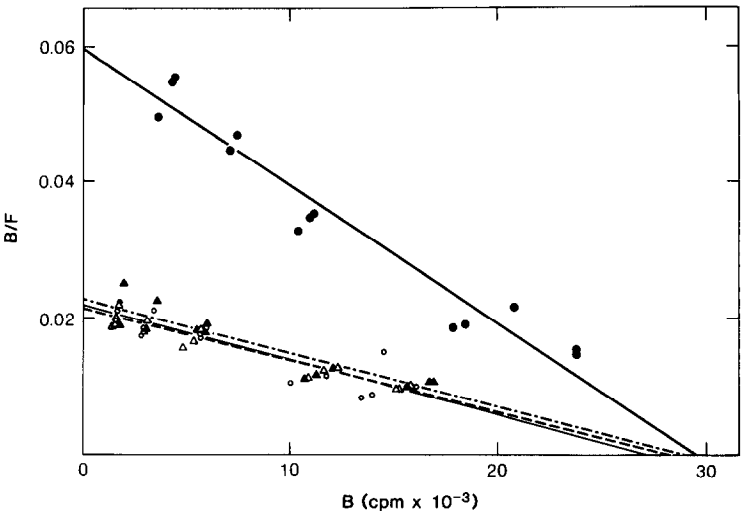

Fig. 2. Additive effects of methylprednisolone and ibuprofen on the affinity and number of FMLP receptors expressed on the PMN surface. Specific [^{3}H]FMLP binding to human neutrophilis is shown at various [^{3}H]FMLP concentrations at 0° in the absence (–●–) and presence of 0.125 mg/ml MP (--△--), 0.125 mg/ml IB (–○–), and 0.0625 mg/ml MP plus 0.0625 mg/ml IB (--▲--). Data are plotted according to Scatchard as (B/F) versus (B) in dpm per 5×10^6 cells, where B = concentration of receptor bound [^{3}H]FMLP and F = concentration of free [^{3}H]FMLP. Correlation coefficients were 0.967 (control), 0.894 (IB), 0.964 (MP), and 0.905 (MP–IB mixture). The derived K_{eq} values were $2.5 \times 10^7\ M^{-1}$ (control), $1.0 \times 10^7\ M^{-1}$ (IB), $9.2 \times 10^6\ M^{-1}$ (MP), and $9.7 \times 10^6\ M^{-1}$ (MP–IB mixture). The derived numbers of receptors per cell were 29,400 (control), 27,200 (IB), 28,000 (MP), and 28,800 (MP–IB mixture). Each point represents the mean of triplicate determinations. A duplicate experiment gave similar results.

peptides [33, 38], as well as that observed for the association of insulin with its receptor on membranes [39].

Reversibility of inhibitor effect. When granulocytes were incubated with 2 mg/ml IB and then washed three times with HBSS-BSA, the inhibition of aggregation as well as the blockade of FMLP binding were both reversed. Granulocyte aggregation induced by activated complement or by FMLP was inhibited by 2 mg/ml IB as previously described [24]. When these cells were washed three times in HBSS-BSA to remove the drug, the addition of activated complement to a stirred suspension of granulocytes in an aggregometer produced a wave of aggregation like that of control granulocytes not exposed to drug (data not shown).

FMLP binding to granulocytes was inhibited markedly by 1.5 mg/ml IB. When these granulocytes were incubated with drug for 2 min at 22° and then washed three times with PBS-BSA at 0°, FMLP binding was similar to that of control cells incubated with PBS (data not shown). Similar reversibility of drug effect was seen when cells were preincubated with drug for 30 min at 22° (not shown).

DISCUSSION

Corticosteroids and the newer nonsteroidal compound, IB, are widely used for their anti-inflammatory effects. Corticosteroids, and more recently IB, have been suggested to be of benefit in a variety of disorders, including endotoxic shock and the adult respiratory distress syndrome [1, 11, 12, 18, 20–22, 40–42]. These benefits, however, have been reported to occur only when the drugs are administered in doses much larger (e.g. 30 mg/kg i.v. for MP) than those conventionally used to achieve an anti-inflammatory effect [17, 18, 41].

The effects of these drugs at such high doses might well be different from those observed with conventional low doses. We have reported previously that such high concentrations of corticosteroids (0.1 to 1 mg/ml) inhibit binding of the chemotactic peptide FMLP to its receptor on the granulocyte surface and that the dose–response relationship of the inhibition of FMLP binding parallels the inhibition of granulocyte responses to FMLP [23]. Similar concentrations of IB (0.1 to 1 mg/ml) also inhibit granulocyte responses to the chemotactic peptides C5a and FMLP, and IB and MP are synergistic in their inhibition of certain granulocyte responses to these stimuli [24]. We have now examined the effect of IB, alone and in combination with MP, on the binding of FMLP to its receptor on human granulocytes. FMLP was used as a probe of granulocyte receptor–ligand interaction because previous *in vitro* and *in vivo* studies of granulocyte responses to chemotactic stimuli suggested FMLP to be a reasonable model for C5a-induced granulocyte leukostasis and tissue injury, and because radiolabeled FMLP is readily available [9, 23, 26, 33, 43].

The mechanism by which peptide binding was inhibited by IB was further clarified by studying the kinetics of the peptide–receptor interaction in live human granulocytes. IB displaced bound radiolabeled FMLP from granulocytes at a similar rate as an excess of unlabeled FMLP, suggesting that IB did not alter the rate of dissociation of FMLP from its

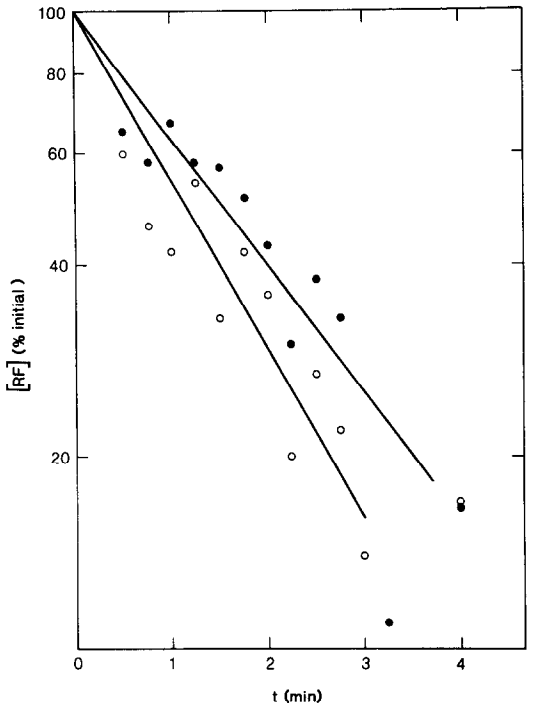

Fig. 3. Effect of ibuprofen on the dissociation rate of the [^{3}H]FMLP–receptor interaction. Dissociation kinetics of the [^{3}H]FMLP–receptor interaction was determined at 0° as described in the text. A representative plot of ln [specific [^{3}H]FMLP binding] (expressed as percent initial binding) corrected for non-dissociating counts, versus time following addition of excess unlabeled FMLP (○) or 5 mg/ml ibuprofen (●), is given. Each point is derived from triplicate samples; k, calculated is 1.0×10^{-2} sec^{-1} in the presence of excess unlabeled FMLP and 0.8×10^{-2} sec^{-1} in the presence of 5 mg/ml ibuprofen.

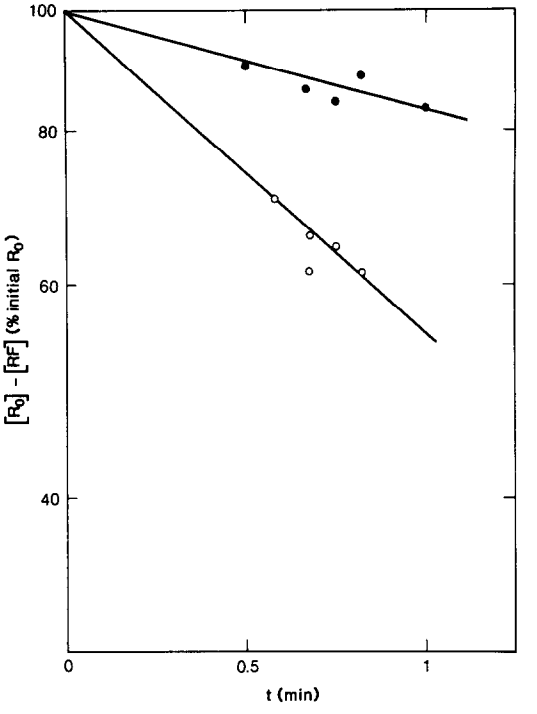

Fig. 4. Ibuprofen effect on the kinetics of the [^{3}H]FMLP–receptor interaction. Association kinetics of a pseudo-first-order association reaction of [^{3}H]FMLP with neutrophil surface receptors at 0° are plotted as $\ln([R_0] - [RF])$ (expressed as percent initial $[R_0]$) versus time; $[R_0]$ = initial receptor concentration (determined by equilibrium studies with saturating [^{3}H]FMLP concentrations), and $[RF]$ = concentration of [^{3}H]FMLP–receptor complexes. Calculated k_f in the absence (○) and presence (●) of 0.125 mg/ml ibuprofen is 2.7×10^5 and 8.6×10^4 l/mol-sec respectively. Each point is the mean of triplicate samples.

surface receptor. In contrast, when the association rate was examined, we found that 0.125 mg/ml IB decreased k_f by approximately 70%. We have reported previously a similar observation using MP [23]. Scatchard analysis of equilibrium binding data revealed that IB, like MP, decreased the affinity of the granulocyte's receptor for the ligand without significantly altering the number of receptors available for binding. IB and MP exhibited similar potency in the inhibition of FMLP binding to granulocytes when equal concentrations of IB and MP were added simultaneously. In contrast to the synergistic effects of MP and IB on granulocyte aggregation, we found no evidence of synergism in their effects on the FMLP-receptor–ligand interaction.

The mechanism by which IB and corticosteroids exert their effect on the receptor–ligand interaction remains unknown. Possible mechanisms controlling receptor affinity suggested by other systems include alterations in membrane fluidity [44, 45], changes in guanine nucleotide levels [46], and transmethylation reactions [47]. Several other drugs, including phenylbutazone, sulfinpyrazone, and the polyene antibiotic amphotericin B (that binds membrane cholesterol), have also been shown to decrease the affinity of the human neutrophil chemotactic peptide receptor for FMLP [48, 49] by unclear mechanisms. Reports of the isolation and characterization of the FMLP receptor have only recently been described [50–54], and it is not known whether these drugs alter the interaction of peptide with solubilized receptor. Our previous report of a synergistic effect of IB and MP in inhibiting some granulocyte responses to FMLP, coupled with our current findings, suggest that these drugs likely have other effects in addition to receptor blockade.

We conclude that high-dose IB, like high-dose corticosteroids, can inhibit granulocyte responses to chemotactic stimuli. These same concentrations of IB and corticosteroids inhibit binding of the synthetic chemotaxin [^{3}H]FMLP to its specific receptor on the granulocyte surface. This inhibition of binding is due primarily to a decrease in the association rate constant for peptide–receptor interaction without a significant change in dissociation rate or receptor number. A marked effect on the FMLP–receptor interaction is seen at a drug concentration of 1 mg/ml, a level approximating the plasma concentration immediately after a 30 mg/kg i.v. bolus in experimental animals, and a prominent effect is seen even

at a 10-fold lower concentration. Thus, these effects are seen even at plasma IB concentrations achieved in patients on more conventional doses of IB given orally (up to 400 μg/ml) [55]. Alteration of cellular receptors may therefore be an important mechanism of action of high-dose IB as well as corticosteroids on granulocyte aggregation. Alteration of other receptor–ligand interactions may also contribute to the reported efficacy of high doses of these drugs in other syndromes.

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