

Cyclosporin H is a potent and selective competitive antagonist of human basophil activation by N-formyl-methionyl-leucyl-phenylalanine

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Background: Cyclosporin A (CsA) binds with high affinity to cyclophilin, a critical step in the molecular mechanism of action of cyclosporins, whereas cyclosporin H (CsH) has extremely low affinity for cyclophilin. CsH differs from CsA by the substitution of the L-methyl valine at position 11 with its D-isomer.

Methods: We compared the effects of CsA and CsH on the release of preformed (histamine) and de novo synthesized inflammatory mediators (peptide leukotriene C_4) from peripheral blood basophils activated by N-formyl-methionyl-leucyl-phenylalanine (FMLP).

Results: CsH (8 to 800 nmol/L) concentration-dependently inhibited histamine and leukotriene C_4 release from purified and unpurified basophils activated by FMLP, whereas CsA (8 to 800 nmol/L) had little inhibitory effect on histamine release from basophils challenged with FMLP. Inhibition of histamine release from basophils challenged with FMLP was extremely rapid and was abolished by washing the cells (three times) before challenge. CsH (8 to 800 nmol/L) had no effect on the release of histamine caused by C5a, platelet activating factor, monocyte chemotactic activating factor, RANTES, IL-8, bryostatin 1, and phorbol myristate. Preincubation of basophils with granulocyte-macrophage colony-stimulating factor (30 and 100 pmol/L), but not IL-1 β (30 and 100 ng/ml), concentration-dependently reversed the inhibitory effect of CsH on FMLP-induced histamine release. CsH competitively inhibited the effect of FMLP on histamine release from basophils. The dissociation constant (K_d) for the CsH-FMLP receptor complex was approximately 9×10^{-8} mol/L, more than 10-fold lower than that ($\cong 1.3 \times 10^{-6}$ mol/L) of N-t-BOC-methionyl-L-leucyl-phenylalanine (BocMLP), a known formyl peptide receptor antagonist. CsH inhibited tritiated FMLP binding to human polymorphonuclear leukocytes with a concentration required to inhibit binding by 50% of approximately 5.4×10^{-7} mol/L, whereas BocMLP was less potent with a concentration required to inhibit binding by 50% of approximately 9.1×10^{-5} mol/L. Scatchard analysis revealed that the decreased tritiated FMLP binding caused by CsH was due to a decrease in the B_{max} (0.22 ± 0.04 nmol/L/ 5×10^6 cells vs 0.09 ± 0.01 nmol/L/ 5×10^6 cells; $p < 0.05$), without a significant difference in the K_d (5.16 ± 1.22 nmol/L vs 6.32 ± 2.42 nmol/L; $p = NS$).

Conclusions: CsH is a potent and selective inhibitor of mediator release from basophils induced by activation of the formyl peptide receptor; it acts by interfering with agonist binding to FMLP receptors. (*J Allergy Clin Immunol* 1996;98:152-64.)

Key words: Basophils, cyclophilin, cyclosporin A, cyclosporin H, histamine, leukotriene C_4

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Supported in part by grants from the National Research Council (C.N.R.) (Targeted Project F.A.T.M.A.; Subproject Prevention and Control of Disease Factors SP. 2; No. 94.00607.PF41 and 95.00856.PF41) and Ministero Università Ricerca Scientifica e Tecnologica, Rome, Italy.

Received for publication July 28, 1995; revised Oct. 9, 1995; accepted for publication Oct. 10, 1995.

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0091-6749/96 \$5.00 + 0 1/1/69883

Basophil granulocytes, normally found in peripheral blood, can infiltrate several extravascular tissues in a variety of pathologic conditions.¹⁻⁵ Basophils and mast cells are the only cells that both synthesize histamine and possess high-affinity receptors for IgE (Fc ϵ RI).^{6,7} Basophils have long been considered "inflammatory cells," but increasing evidence indicates that through the elaboration of various cytokines (e.g., IL-4, IL-13) and the expression of the CD40 ligand, they play a more complex role than originally thought.^{8,9} Circulating and tissue infiltrates of basophils play a role in

Abbreviations used

BocMLP:	N-t-BOC-methionyl-L-leucyl-phenylalanine
CsA:	Cyclosporin A
CsH:	Cyclosporin H
FMLP:	N-formyl-methionyl-leucyl-phenylalanine
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
HPLC:	High-performance liquid chromatography
IC ₅₀ :	Concentration required to inhibit binding by 50%
LT:	Leukotriene
MCAF:	Monocyte chemotactic activating factor
PAF:	platelet activating factor
PBS:	Phosphate-buffered saline
PIPES:	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PMNs:	Polymorphonuclear neutrophils
TPA:	12-O-Tetradecanoyl-phorbol-13-acetate

several allergic,¹⁰⁻¹² inflammatory,^{5,10} and chronic disorders affecting various organs.^{5,13}

Basophils can be activated to secrete proinflammatory mediators and cytokines by cross-linking surface-bound IgE or FcεRI.^{8,14} This can be accomplished by specific antigens¹⁵; such bacterial products as protein A¹⁶ and protein L,¹⁷ naturally occurring anti-IgE,¹⁸ and antibodies against the α chain of the FcεRI.¹⁹

These stimuli are bi- or multivalent and induce a series of biochemical events resulting in the secretion of preformed mediators (e.g., histamine), the de novo synthesis of lipid mediators (e.g., peptide leukotriene C₄ [LTC₄])²⁰ and IL-4,⁸ and the expression of CD40 ligand.⁹ In addition to multivalent antigen-IgE-mediated stimuli, human basophils can be activated by a variety of non-IgE-dependent monovalent stimuli such as N-formyl-methionyl-leucyl-phenylalanine (FMLP),²¹ C5a,^{22,23} monocyte chemotactic activating factor (MCAF),²⁴ RANTES,²⁵ IL-8,²⁶ and platelet activating factor (PAF).²⁷ These stimuli activate different receptors that belong to the G protein-coupled receptor superfamily and mediate chemotaxis, degranulation, and respiratory burst.²⁸ Because inflammatory and immune reactions are not necessarily characterized by antigen-driven reactions, these stimuli may also play a role in several pathophysiologic conditions. Among these stimuli, N-formy-

lated peptides (FMLP) released from bacteria^{29,30} and also from mitochondria³¹ are potent stimuli for human basophils.²¹ Biochemical and pharmacologic studies suggest significant differences in the signal transduction pathways underlying the activation caused by the aggregation of cell-surface IgE and the activation of distinct univalent receptors by FMLP,³² C5a,³³ PAF,²⁷ MCAF,²⁴ RANTES,²⁵ and IL-8.²⁶

Given the relevance of human basophils and their mediators to immune and inflammatory reactions, their pharmacologic modulation is of considerable importance. We have recently demonstrated that the immunosuppressive compound cyclosporin A (CsA), a cyclic undecapeptide extracted from *Tolypocladium inflatum* Gams,³⁴ is a potent inhibitor of the IgE-mediated activation of human basophils in vitro, as well as in vivo.³⁵⁻³⁷ CsA exerts its antiinflammatory activity, presumably by binding to cytoplasmic cyclophilins,^{36,38} which function as specific receptors³⁹ for this class of compounds.

Several natural and synthetic structural congeners of CsA with substitutions on the ring structure have been identified or synthesized. CsH is a natural analog of CsA in which D-methylvaline is substituted for L-methylvaline at position 11.⁴⁰ CsH showed no immunosuppressive effects in a variety of systems,⁴¹⁻⁴⁴ and this inactivity has been attributed to its low affinity for cyclophilin.^{36,41} Pharmacologic concentrations of CsA inhibit antigen- and anti-IgE-induced release of mediators (histamine, LTC₄, and/or prostaglandin D₂) from human basophils^{35,36,38} and mast cells.⁴⁵ In contrast, CsH did not inhibit the immunologic release of mediators from human FcεRI⁺ cells.^{36,45}

These findings prompted us to compare the effects of CsA and CsH on the release of preformed (histamine) and de novo synthesized mediators (LTC₄) from basophils challenged with monovalent stimuli such as FMLP, C5a, PAF, MCAF, RANTES, and IL-8. We also examined the effects of interactions between CsH and various cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-1β, on the release of mediators from basophils.

METHODS

Reagents

The following were purchased: C5a and N-t-BOC-methionyl-L-leucyl-phenylalanine (BocMLP) (Sigma Chemical Company, St. Louis, Mo.); N-formyl-methionyl-leucyl-L-phenylalanine (FMLP) (Calbiochem-

Behring Co., La Jolla, Calif.); PAF C_{16:0} (Biomol, Philadelphia, Pa.); 12-0-tetradecanoyl-phorbol-13-acetate (TPA) (P-L Biochemicals Inc., Milwaukee, Wis.); dextran T 70 (Pharmacia Fine Chemicals, Uppsala, Sweden); tritiated LTC₄ (35.7 Ci/mmol; New England Nuclear, Boston, Mass.); tritiated FMLP (40.5 Ci/mmol; Du Pont, De Nemours, Belgium). Rabbit anti-human IgE-Fc ϵ antibody was a generous gift from Drs. Teruko and Kimishige Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, Calif.). Synthetic LTB₄, LTC₄, LTD₄, and LTE₄ and the rabbit anti-LTC₄ antiserum were kind gifts from Dr. J. Rokach. Bryostatin 1 was generously donated by Dr. G. R. Pettit (Arizona State University, Cancer Research Institute, Temple, Ariz.). Recombinant human IL-1 β was kindly donated by Dr. D. Boraschi (Sclavo Research Center, Siena, Italy). Recombinant human MCAF was a gift from Dr. S. Sozzani (Istituto Ricerche Farmacologiche "Mario Negri", Milan, Italy). Recombinant human RANTES and IL-8 were gifts from Dr. C. Stellato (Johns Hopkins University, Baltimore, Md.). Recombinant human GM-CSF was obtained from Sandoz Ltd. (Vienna, Austria). CsA, CsH, and cyclosporins C, D, and G were gifts from Dr. D. Romer and Dr. E. Rissi (Sandoz Ltd., Basel, Switzerland).

Buffers

Buffer P consisted of 110 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L piperazine-N-N'-bis(2-ethanesulfonic acid) (PIPES), pH 7.4. Buffer PC consisted of buffer P with the addition of 1.0 mmol/L CaCl₂. Phosphate-buffered saline (PBS) contained: NaCl, 8.0 gm/L; Na₂HPO₄ · 7H₂O, 2.89 gm/L; KH₂PO₄, 0.2 gm/L and KCl, 0.2 gm/L (pH 7.3).¹⁵

Preparation of peripheral blood leukocytes containing basophils and basophil purification

The study protocol was approved by the Ethics Committee for Human Research of the University of Naples Federico II School of Medicine. Informed consent was obtained from normal subjects, and venous blood was drawn into tubes containing a final concentration of 0.008 mol/L ethylenediaminetetraacetic acid and 1.1% dextran T 70. Erythrocytes were allowed to settle for 90 minutes at 22° C. The leukocyte-rich upper layer was drawn off, pelleted (200 g, 8 minutes, 4° C), and washed twice in buffer P, as previously described.¹⁵ This preparation, which generally contains about 1% basophils, is hereafter referred to as *basophils*. Contaminating cells were lymphocytes, neutrophils, eosinophils, monocytes, and platelets. Purification of basophils was performed by a modification of the method described by de Boer and Roos.⁴⁶ In brief, "buffy coat" cell packs from healthy volunteers were reconstituted in PBS containing 0.5 gm/L human serum albumin and 3.42 gm/L sodium citrate and loaded onto a countercurrent elutriator (model J2-21; Beckman Instruments, Inc., Fullerton,

Calif.). Several fractions were collected, and fractions containing basophils in large numbers and of improved purity (>15%) were further enriched by discontinuous Percoll gradients (Pharmacia Fine Chemicals). With this technique, yields ranged from 3 to 8 × 10⁶ basophils, and purities ranged from 35% to 75%,²³ as determined by basophil staining with alcian blue and counting in a Spiers-Levy eosinophil counter (C. A. Mausser & Son, Philadelphia, Pa.).⁴⁷

Histamine release assay

In experiments with basophils, approximately 6 × 10⁴ purified or unpurified basophils (per tube) were placed in 12 × 75 mm polyethylene tubes (Sarstedt Inc., Princeton, N.J.) and warmed to 37° C; 0.2 ml of each prewarmed releasing stimulus was added, and incubation was continued at 37° C. At the end of this step, the reaction was stopped by centrifugation (1000 g, 22° C, 2 minutes), and the cell-free supernatants were stored for assay of histamine⁴⁸ and LTC₄ content.⁴⁹ Total histamine content was assessed by lysis induced by incubating the cells and supernatants with HClO₄ (final concentration, 2%) before centrifugation. All values are based on means of duplicate or triplicate determinations. Replicates differed in histamine content by less than 10%. Unless stated otherwise, cells were preincubated for 5 minutes with CsA and CsH before addition of the stimuli; preincubation time was not crucial to the experiment, and the test tubes could be handled within 5 minutes. Unless indicated otherwise, cells were challenged with an optimal concentration of anti-IgE, FMLP, C5a, PAF, MCAF, RANTES, IL-8, TPA, or bryostatin 1. At the end of the incubation, the cell-free supernatants were assayed for histamine with an automated fluorometric technique.⁴⁸ To calculate histamine release as a percentage of total cellular histamine, the "spontaneous" release of histamine from basophils (0% to 10% of the total cellular histamine) was subtracted from both numerator and denominator.⁴⁵ All values are based on means of duplicate or triplicate determinations. Replicates differed from each other in histamine content by less than 10%.

High-performance liquid chromatography and radioimmunoassay of LTC₄

A Beckman model 420 liquid chromatograph was used for high-performance liquid chromatography (HPLC). A Waters C₁₈ column (Waters Associates, Milford, Mass.) was used to separate arachidonic acid metabolites.⁴⁹ HPLC was done by using mixtures of water/acetonitrile/methanol/acetic acid 60%/33.6%/5.4%/1.0%, adjusted to pH 5.6 with 10% NH₄OH; flow rate was 1 ml/min. The detector was set to read at a wavelength of 280 nm with a full-scale absorbance setting of 0.005 units. All HPLC runs were done first with LT standards to standardize retention time, then the column was given a "clean" run without sample, after which a sample was run. In some experiments

tritiated LTC₄ was used for time standardization; fractions were collected at 1-minute intervals and concentrated to dryness with a solvent concentrator. In this case 4.5 ml of a toluene-based scintillation cocktail was added, and radioactivity was measured by scintillation spectrometry with a Tri-Carb 1600 TR (Packard, Meriden, Conn.). When the samples were to be assayed by HPLC, the supernatants were extracted and concentrated. C₁₈ Sep-Pak cartridges (Waters Associates) were pre-wetted with 2 ml of methanol (100%) and 5 ml of water. Supernatants (1 ml) were applied to the column, after which the cartridges were washed with 5 ml of 20% methanol. LTs were eluted with 1 ml of 100% methanol; the methanol was evaporated to dryness under a nitrogen stream; the residue was dissolved in 0.1 ml of the mobile phase and finally loaded onto a 20 μ l sample loop for chromatography. Recoveries of LTs with this technique ranged from 65% to 95%, calculated by using prostaglandin B₂ (100 ng) as internal standard. LTC₄ concentrations were established by computer analysis of peaks or by radioimmunoassay, as described previously.⁴⁹

Purification of human neutrophils and tritiated FMLP binding assay

Neutrophils were purified from peripheral blood obtained from normal subjects, as previously described.²⁰ The resulting cell preparation contained approximately 99% polymorphonuclear neutrophils (PMNs); platelet contamination was always less than 1%, as determined by direct counting. Cell preparations consisted of more than 96% viable neutrophils as revealed by trypan blue dye exclusion. The binding of tritiated FMLP was measured essentially as described by Naccache et al.⁵⁰ with minor modifications. Briefly, PMNs ($\approx 5 \times 10^6$ /ml) were incubated in PBS at 37° C for 15 minutes in the presence or absence of increasing concentrations of CsH, CsA, and BocMLP. Subsequently, the cells (in duplicate for each experiment) were immediately placed in an ice bath, and tritiated FMLP was added to aliquots of neutrophils in the presence or absence of unlabeled FMLP (10^{-4} mol/L). Binding was allowed to reach equilibrium by incubation for 60 minutes at 4° C. Bound and free tritiated FMLP were separated by centrifugation of the cells (1 minute, 12,000 g) through a layer of silicon oil (Versilube F 50; General Electric Silicones, Milano, Italy). Specific binding was determined from the difference in the radioactivity recovered from the pellet fractions in the absence and presence of excess unlabeled FMLP.

Statistical analysis

The results are expressed as means $\pm$ SEM. Concentration required to inhibit binding by 50% (IC₅₀) values were compared, after log transformation, by means of paired or unpaired Student's *t* test. The level of statistical significance was *p* < 0.05. The data subjected to linear regression analyses were calculated by the least-squares method ($y = a + bx$) in which *a* was the y-axis intercept and *b*, the slope of the line.⁵¹

RESULTS

Effect of CsH and CsA on FMLP-induced histamine release from human basophils

In a first series of experiments we assessed the effects of increasing concentrations of CsA and CsH on histamine release from human basophils induced by FMLP. The concentrations of cyclosporins used (8 to 800 nmol/L) included those reached in vivo during CsA treatment (8 to 300 nmol/L)³⁷ and did not induce spontaneous lactate dehydrogenase or histamine release from basophils (data not shown). Fig. 1, *A*, shows the results from 11 experiments in which basophils were preincubated (5 minutes at 37° C) with different concentrations of CsA or CsH before challenge with FMLP. CsH caused concentration-dependent inhibition of histamine release, ranging from 15% at 8 nmol/L to approximately 90% at 800 nmol/L. Maximally effective concentrations of CsH inhibited FMLP-mediated histamine release by about 90%, whereas the same concentrations of CsA caused no more than 20% to 30% inhibition when basophils were activated by FMLP. CsH was more potent than CsA when the formyl peptide receptors on basophils were activated by FMLP (IC₅₀ = 154.5 ± 36.6 nmol/L vs > 1000 nmol/L; *p* < 0.001). The maximal inhibitory effect of CsH was also significantly higher than that of CsA ($90.6\% \pm 3.7\%$ vs $27.1\% \pm 6.3\%$; *p* < 0.001). In the same group of experiments we confirmed³⁶ that CsH had no effect on the release of mediators from basophils caused by IgE cross-linking, whereas CsA (8 to 800 nmol/L) concentration-dependently inhibited the release reaction (Fig. 1, *B*). Other cyclosporins (including cyclosporins C, D, and G) had little or no effect on basophil mediator release induced by FMLP (data not shown).

To exclude the possibility that contaminating cells were responsible for the inhibitory effect of CsH on the activation of basophils by FMLP, experiments were carried out with enriched preparations of basophils. The results presented in Table I indicate that the percent of purified basophils (from 38% to 78%) does not influence the inhibitory effect of CsH on the activation of the FMLP receptor.

Effect of CsH on basophil histamine release induced by C5a, PAF, MCAF, RANTES, IL-8, TPA, and bryostatine 1

The results of this study and our previous findings³⁶ indicate that CsA and CsH exert strikingly different inhibitory effects on the release of mediators from human basophils, depending on the

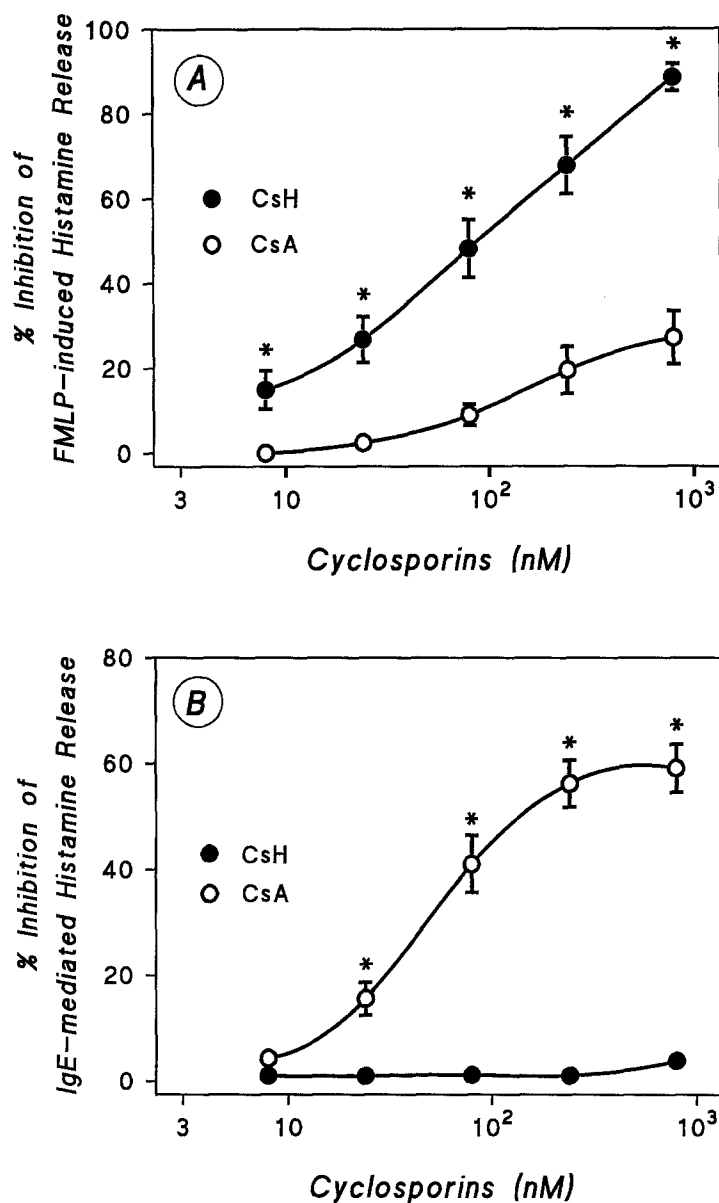


FIG. 1. A, Effect of various concentrations of CsH (filled circles) and CsA (open circles) on histamine release from human basophils induced by FMLP. Cells were preincubated (5 minutes at 37° C) with CsH or CsA and then incubated (30 minutes at 37° C) with optimal concentrations of FMLP. The percent histamine release caused by FMLP from basophils preincubated with CsH was $48.0\% \pm 6.1\%$, and with CsA, $21.8\% \pm 4.2\%$. Results are expressed as means $\pm$ SEM of percentage inhibition caused by CsH or CsA (except when too small to depict) from 11 experiments. * $p < 0.001$ compared with the corresponding group. **B,** Effect of various concentrations of CsH (filled circles) and CsA (open circles) on histamine release from human basophils induced by anti-IgE. Cells were preincubated (5 minutes at 37° C) with CsH or CsA and then incubated (30 minutes at 37° C) with optimal concentrations of anti-IgE. The percent histamine release caused by anti-IgE from basophils preincubated with CsH was $24.0\% \pm 2.3\%$, and with CsA, $22.4\% \pm 3.2\%$. Results are expressed as means $\pm$ SEM of percentage inhibition caused by CsH or CsA (except when too small to depict) from nine experiments. * $p < 0.001$ compared with the corresponding group.

nature of the activating stimulus. We therefore examined the effects of CsH on histamine release from basophils induced by such monovalent stimuli as C5a and PAF, which activate distinct G

protein-coupled receptors, independent of the IgE and FMLP receptors.^{22, 27} Preincubation (5 minutes at 37° C) of basophils with different concentrations of CsH (8 to 800 nmol/L) did not cause

TABLE I. Effect of various concentrations of CsH on histamine release from human basophils of different purities challenged with FMLP

	Experiment (purity of basophils)		
	1 (38%)	2 (78%)	3 (69%)
Percent histamine release			
No drug	33	44	55
CsH (nmol/L)			
24	25	32	51
80	20	26	44
240	11	15	38
800	2	6	12

Cells were preincubated for 5 minutes with CsH and then incubated (30 minutes at 37° C) with optimal concentrations of FMLP (10^{-7} mol/L). Results are expressed as means of duplicate samples.

any inhibitory effect on the release of histamine from basophils induced by C5a ($n = 2$) and PAF ($n = 2$) (data not shown). In a series of three experiments we also examined the effects of CsH on the activation of basophils by C-x-C (IL-8) and C-C chemokines (MCAF and RANTES), which activate distinct receptors.²⁴⁻²⁶ In these experiments CsH (800 nmol/L) did not influence the releasing activity of MCAF, RANTES, and IL-8 (data not shown). Similar results were obtained when cells were preincubated with CsH (8 to 800 nmol/L) before challenge with bryostatin 1 ($n = 2$) and TPA ($n = 2$), which induce mediator release from basophils,^{52, 53} presumably by directly activating different isoforms of protein kinase C^{53, 54} (data not shown).

Effects of CsH on the de novo synthesis of LTC₄ from human basophils induced by FMLP

Activation of formyl peptide receptors by FMLP causes the release of LTC₄ from human basophils.⁵⁵ Peptide LTs exert various proinflammatory, vasoactive, and immunoregulatory effects and have been implicated in the pathogenesis of several immune and inflammatory disorders.^{56, 57} The pharmacologic modulation of newly synthesized eicosanoids from basophils and mast cells does not always parallel that of preformed mediators.⁵⁸ Table II shows the results of three of six experiments in which CsH (24 to 800 nmol/L) inhibited the de novo synthesis of LTC₄ from enriched preparations of basophils (from 45% to 85%) activated by FMLP.

TABLE II. Effect of various concentrations of CsH on the de novo synthesis of LTC₄ from human basophils challenged with FMLP

	Experiment (purity of basophils)		
	1 (45%)	2 (61%)	3 (85%)
LTC ₄ (ng/10 ⁶ basophils)			
No drug	69.0	43.9	143.5
CsH (nmol/L)			
24	40.7	38.8	145.6
80	38.5	23.2	99.4
240	31.8	24.9	67.4
800	22.3	9.8	15.8

Cells were preincubated for 5 minutes with CsH and then incubated (30 minutes at 37° C) with optimal concentrations of FMLP (10^{-7} mol/L). Results are expressed as means of duplicate samples.

The inhibition ranged from about 15% at 24 nmol/L to 80% at 800 nmol/L.

Reversibility and kinetics of the inhibitory effect of CsH on histamine release from human basophils induced by FMLP

CsA acts on basophils very rapidly, and its inhibitory effect is irreversible because it was not reduced after washing the leukocytes (three times).³⁶ The inhibition of FMLP-induced histamine release caused by CsH was also rapid, being unaffected by preincubation times from 2 to 20 minutes (data not shown). In contrast, in six experiments the maximum inhibitory effect of CsH (8 to 800 nmol/L) on histamine release induced by FMLP from basophils was reversible because it was significantly reduced after washing the leukocytes (three times). The percent inhibition decreased, in fact, from $74.0\% \pm 5.2\%$ to $21.0\% \pm 5.3\%$; ($p < 0.005$) (Fig. 2).

Interaction between CsH or BocMLP and FMLP-induced histamine release from basophils

In a series of experiments we examined how CsH or BocMLP, a competitive antagonist of the FMLP receptor,⁵⁹ affects FMLP-induced histamine release from basophils. BocMLP binds reversibly to the FMLP receptor but has no intrinsic releasing or chemotactic activity.⁵⁹ Fig. 3 shows the results obtained in one of eight typical experiments. As previously shown,⁵⁹ BocMLP (2 to 15 μ mol/L) concentration-dependently inhibited FMLP-induced histamine release. Preincubation of basophils with increasing concentrations of BocMLP shifted the concentra-

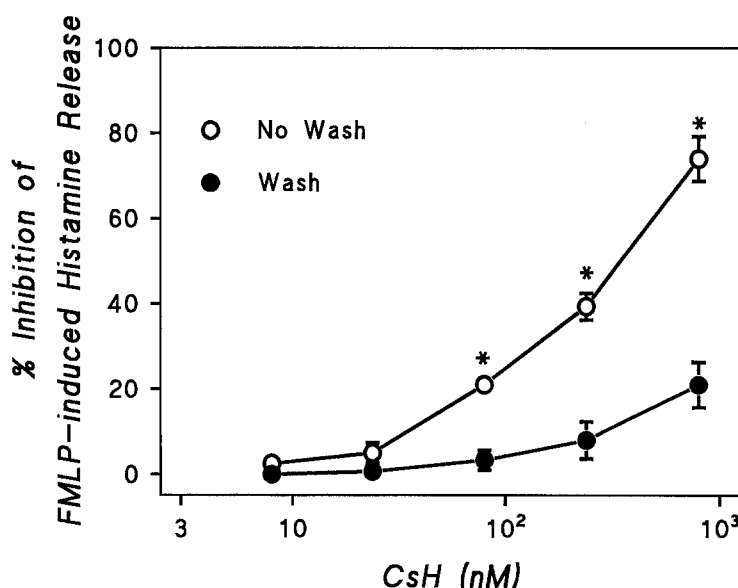


FIG. 2. Effect of cell washing on the inhibition of FMLP-induced histamine release from basophils caused by CsH. Two sets of enriched preparations of human basophils (from 6% to 42%) were preincubated (5 minutes at 37° C) with CsH or buffer. The two sets of cells were washed (three times) in PIPES buffer (1 ml, +4° C) and resuspended in PIPES buffer containing 1 mmol/L CaCl₂. Cells previously incubated in buffer were incubated (5 minutes at 37° C) with CsH, whereas cells previously incubated in the presence of CsH were incubated for 5 minutes in the absence of the drug. Then the two sets of cells were challenged with optimal concentrations of FMLP, and release was allowed to proceed for 30 minutes at 37° C. Results are expressed as means $\pm$ SEM of percentage inhibition caused by CsH (except when too small to depict) in six experiments. * $p < 0.001$ compared with the corresponding group.

tion-response curve caused by FMLP to the right. In parallel experiments, one of which is illustrated in Fig. 3, *B*, preincubation of basophils with increasing concentrations of CsH (80 to 800 nmol/L) shifted the concentration-response curve caused by FMLP to the right. The shift of concentration-response curves of FMLP-induced histamine release to the right and the absence of depression of the maximum effect of FMLP in the presence of CsH or BocMLP suggested that CsH could be a competitive antagonist of FMLP. Therefore we measured the affinity constant of the CsH-receptor interaction. Assuming that CsH and FMLP compete for a receptor site and that the CsH-R interaction is freely reversible, it follows that:



in which the CsH-R complex inhibits histamine release and the FMLP-R complex promotes histamine release. At equilibrium, it can be shown⁶⁰ that $(x - 1) = K_d$, in which K_d is the dissociation constant of the CsH-R complex and x is the ratio of the FMLP concentrations in the presence and absence of CsH required to produce the same

effect. Plotting the logarithm of $(X - 1)$ against the logarithm of CsH concentrations on a Schild plot provides, in the case of a competitive inhibitor, a straight line with a slope of 1 and an intercept on the abscissa corresponding to $-\log K_d$. Experiments such as that shown in Fig. 3 were used to construct a Schild plot,⁶⁰ and it was found that the slopes were not significantly different from 1. Fig. 4 shows the least-squares fit to the experimental points from three (CsH) or five (BocMLP) separate experiments. In these experiments the mean $\pm$ SEM value for K_d of the CsH-FMLP receptor complex was $9.0 \times 10^{-8} \text{ mol/L} \pm 3.0 \times 10^{-8} \text{ mol/L}$, and for the BocMLP complex the mean $\pm$ SEM value was $1.3 \times 10^{-6} \text{ mol/L} \pm 0.1 \times 10^{-6} \text{ mol/L}$. The latter value was significantly higher than that obtained with CsH ($p < 0.05$), suggesting that CsH is a more potent inhibitor of the FMLP receptor on human basophils than BocMLP.

Effect of CsH, BocMLP, and CsA on tritiated FMLP binding to human leukocytes

Our results indicate that the CsH inhibition of FMLP-mediated activation of basophils is rapid,

reversible, and specific for the FMLP receptor. The results of the Schild plot analysis suggest that the inhibition by CsH of the FMLP-induced mediator release could be due to interference with the binding of FMLP to its receptor in basophils. Binding studies of tritiated FMLP in basophils would be ideal, but they require large numbers of highly purified cells that are not currently available. We therefore compared the effects of CsH, CsA, and BocMLP on tritiated FMLP binding to intact human polymorphonuclear neutrophils (PMNs) purified ($\approx 99\%$) from peripheral blood. Fig. 5 shows the results of three experiments. CsH and BocMLP inhibited the binding of tritiated FMLP to its receptor in intact PMNs. The IC_{50} for CsH (3.6×10^{-7} mol/L $\pm 0.8 \times 10^{-7}$ mol/L) was significantly lower than that of BocMLP ($1.3 \times 10^{-5} \pm 0.1 \times 10^{-5}$ mol/L) ($p < 0.01$), reinforcing the observation that CsH is the most potent receptor antagonist of the FMLP receptor so far identified. The specificity of this observation was supported by the finding that CsA (0.001 to 100 μ mol/L) had no inhibitory effect on tritiated FMLP binding in human PMNs. Scatchard analysis of tritiated FMLP binding to control and CsH-treated (800 nmol/L) neutrophils revealed that the CsH-treated leukocytes had a decreased number of apparent FMLP receptors as determined by a significant decrease in B_{max} (0.22 ± 0.04 nmol/L/ 5×10^6 cells vs 0.09 ± 0.01 nmol/L/ 5×10^6 cells; $p < 0.05$; $n = 3$). The dissociation constant (K_d) of the CsH-treated neutrophils did not vary from that of controls (6.32 ± 2.42 nmol/L vs 5.16 ± 1.22 nmol/L; $p = NS$).

Interactions between GM-CSF or IL-1 β and CsH on FMLP-induced activation of basophils

The observations described above highlight a new antiinflammatory effect of CsH on human basophils. Inflammatory reactions are characterized by overproduction of such cytokines as GM-CSF and IL-1 β , and human basophils possess surface receptors for these cytokines.⁶¹⁻⁶³ Preincubation of basophils with GM-CSF and IL-1 β can modulate basophil activation.^{61, 63} We therefore attempted to determine whether GM-CSF and IL-1 β modulate the inhibitory effect of CsH on FMLP-induced mediator release from basophils. Fig. 6 illustrates one of six typical experiments showing that preincubation (2 hours at 37°C) of low concentrations (30 and 100 pmol/L) of GM-CSF, which alone did not induce histamine release from basophils but enhanced mediator release,

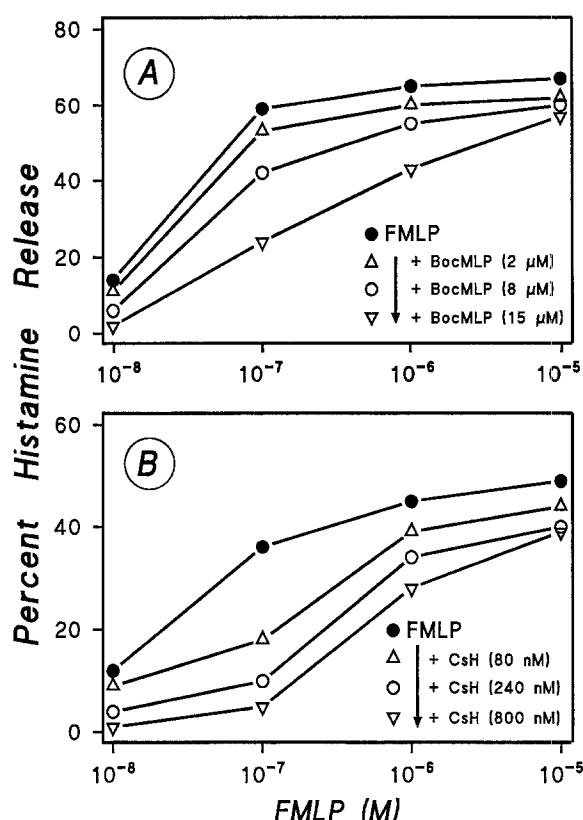


FIG. 3. Effect of various concentrations of BocMLP (A) and CsH (B) on histamine release from purified human basophils challenged with FMLP. The cells were preincubated for 5 minutes with BocMLP or CsH. After 5 minutes, FMLP was added, and the cells were incubated for an additional 30 minutes at 37°C. Each point represents the mean of duplicate determinations from one of three typical experiments.

concentration-dependently reversed the inhibitory effect of CsH on FMLP-induced histamine release from basophils. The specificity of this observation was demonstrated by the results of three experiments, indicating that preincubation (10 minutes to 2 hours at 37°C) with IL-1 β (30 and 100 ng/ml) failed to reverse the inhibitory effect of CsH on FMLP-induced histamine release from basophils (data not shown).

DISCUSSION

This study demonstrates that nanomolar concentrations of CsH exert a potent antiinflammatory effect by inhibiting the release of preformed and de novo synthesized chemical mediators from human basophils activated by FMLP. Our results are compatible with the hypothesis that the inhibitory effect of CsH on the release of mediators

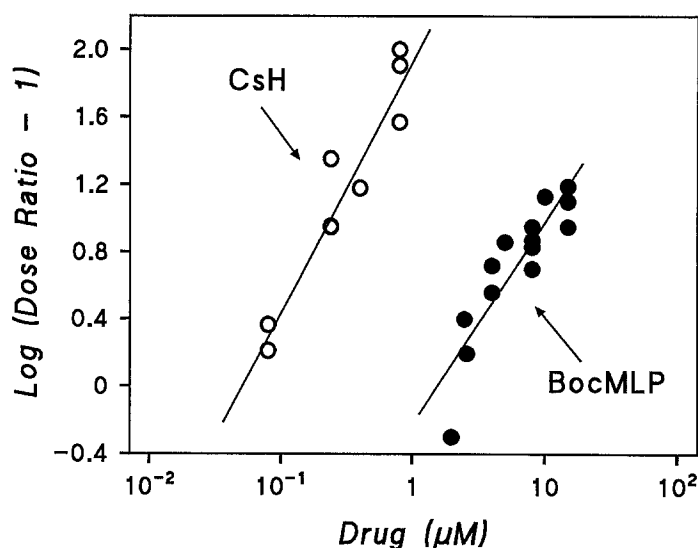


FIG. 4. Schild plot for the antagonism by CsH or BocMLP of the effect of FMLP from experiments such as those shown in Fig. 3. The lines are the least-squares fit to the experimental points from three (CsH) or five (BocMLP) separate experiments. In these experiments the slopes of the fitted lines were not significantly different from 1. The K_d obtained from the value of $\log_{10}$ (CsH) in which $\log_{10}$ (dose-ratio - 1) = 0 was 9×10^{-8} mol/L $\pm 3 \times 10^{-8}$ mol/L. The K_d obtained from the value of $\log_{10}$ (BocMLP) in which $\log_{10}$ (dose-ratio - 1) = 0 was 1.34×10^{-6} mol/L $\pm 0.18 \times 10^{-6}$ mol/L; ($p < 0.05$).

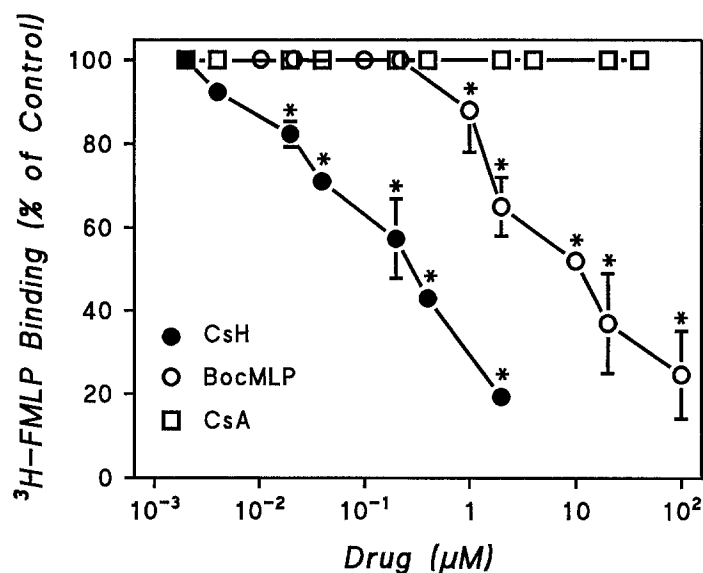


FIG. 5. Effect of various concentrations of CsH, BocMLP, and CsA on FMLP binding (tritiated FMLP, 3 nmol/L) to formyl peptide receptors in human neutrophils. Cells were preincubated (5 minutes at 37° C) with CsH, BocMLP, or CsA. Binding of tritiated FMLP was measured as described in Methods. Results are expressed as the mean $\pm$ SEM of three experiments (except when too small to depict). * $p < 0.01$ when compared with control values.

from basophils is mediated by the competitive interaction at the FMLP receptor level. The inhibitory effect of CsH is reversible and can be reversed by preincubation with GM-CSF, but not with IL-1 β .

CsH is a natural stereoisomer of CsA in which D-methylvaline at position 11 is substituted for L-methylvaline.⁴⁰ This simple modification of the chemical structure markedly affects the immuno-suppressive and antiinflammatory properties of the

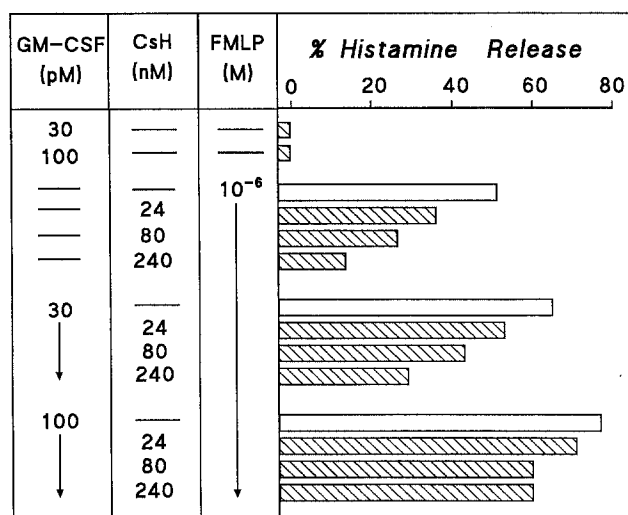


FIG. 6. Reversal of CsH-induced inhibition of FMLP-induced histamine release by GM-CSF. Cells were preincubated (2 hours at 37° C) with GM-CSF (30 and 100 pmol/L) and with CsH (5 minutes at 37° C) and then incubated with FMLP (30 minutes at 37° C) (10^{-7} mol/L). Results are expressed as the mean of duplicate samples from one of three typical experiments.

two compounds. Since its discovery, CsH has been found to lack immunosuppressive activity in several systems.⁴¹⁻⁴⁴ We found that CsH, unlike CsA, had no effect on the release of proinflammatory mediators from human basophils and mast cells activated by IgE cross-linking.^{36, 45} This lack of immunosuppressive and antiinflammatory activities has been generally attributed to its very low affinity for cyclophilin, the main cytoplasmic receptor for cyclosporins and a key molecule in their immunosuppressive and antiinflammatory effects.^{39, 41, 64}

It is therefore likely that the newly identified antiinflammatory activity of CsH is not cyclophilin-mediated but involves competitive antagonism at the level of the FMLP receptor. This is a novel mechanism of antiinflammatory action of CsH, and the possibility that other cyclosporins might interfere with the binding of FMLP receptors on human inflammatory cells cannot be excluded. The specificity of CsH as a competitive antagonist of FMLP receptors versus other receptors is remarkable. We have demonstrated that CsH has no effect on the activation of other G protein-coupled receptors (i.e., C5a and PAF), enzymatic activities (i.e., protein kinase C). Furthermore, CsH does not interfere with the signal transduction on pathways involved in the basophil activation caused by C-x-C (IL-8) and C-C chemokines (MCAF and RANTES). However, the possibility that CsH might interfere with other surface receptors on basophils or on other inflammatory cells cannot be

ruled out. The specificity of CsH as a competitive antagonist of FMLP receptor versus the C5a receptor is intriguing because the formyl peptide receptor is approximately 34% identical in overall sequence to the C5a receptor.²⁸

Although CsH appears to be a selective inhibitor of FMLP-induced activation of human inflammatory cells, its antiinflammatory activity is not limited to human basophils. In fact, CsH is an inhibitor of FMLP-induced superoxide formation by human PMNs.⁶⁵ In addition, CsH interferes with tritiated FMLP binding to membranes of dimethyl sulfoxide- or N⁶,o-dibutyryladenosine 3':5'cyclic monophosphate-differentiated HL-60 cells.⁶⁶ We have extended the latter finding by demonstrating that CsH is a competitive antagonist at the FMLP receptor level on human basophils, with a K_d of approximately 9×10^{-8} mol/L. Binding of tritiated FMLP to intact human PMNs also confirms that CsH, but not CsA, is a potent receptor antagonist of FMLP. Taken together, these findings indicate that CsH is a novel and potent competitive antagonist of the FMLP receptor on human inflammatory cells.

Interestingly, CsH appears to be a very potent FMLP receptor antagonist with a K_d of approximately 9×10^{-8} mol/L in basophils (i.e., 10-fold lower than that of BocMLP, the best FMLP receptor antagonist previously available).⁵⁹ Similarly, the IC_{50} for CsH found in human PMNs is over 10-fold lower than that of BocMLP. These findings emphasize the potency of CsH as a competitive

antagonist for the FMLP receptor on human inflammatory cells and suggest a novel mechanism of antiinflammatory effect for this versatile class of compounds. Scatchard analysis of tritiated FMLP binding to neutrophils revealed that CsH significantly decreased the $B_{\max}$ without affecting the K_d .

CsH appears to be a selective inhibitor of the release of preformed (histamine and lysosomal enzymes) and de novo synthesized (LTC_4 and $O_2^\bullet$) chemical mediators from human basophils and neutrophils induced by activation of the formyl peptide receptor.^{65, 66} Basophil granulocytes and neutrophils, through the release of proinflammatory and vasoactive mediators and of different cytokines, play critical roles in host defense and in several immune^{8, 9} and inflammatory reactions.^{1-4, 10-12} Considering the potency and specificity of CsH, it is feasible that low doses of CsH or analogs with similar pharmacologic properties could be useful in the treatment of diseases caused by cell activation by such bacterial products as FMLP.

An interesting finding of this study is the interaction between the inhibitory effect of CsH and the activity cytokines. GM-CSF, but not IL-1 β , reversed the inhibitory effect of CsH on FMLP-induced activation of basophils. It has been shown that preincubation of human PMNs with GM-CSF induces a rapid increase in high-affinity receptors for FMLP, concomitant with priming for enhanced neutrophil oxidative metabolism.⁶⁷ In our system, preincubation with GM-CSF reversed the inhibitory effect of CsH on formyl peptide-induced histamine release. These observations are compatible with the hypothesis that GM-CSF and CsH exert their opposite effects at the level of the formyl peptide receptor on basophils. The specificity of this observation is supported by the lack of effect of IL-1 β on the inhibitory activity of CsH in human basophils. We can speculate that inflammatory reactions, characterized by overproduction of GM-CSF but not IL-1 β , could overcome the antiinflammatory property of CsH.

It is likely that CsH inhibits histamine release by acting directly on basophils. This hypothesis is supported by the results of the experiments performed with preparations containing various percentages of basophils. In these experiments we found that the potency and efficacy of CsH in the inhibition of FMLP-induced histamine release was unaffected by the percentage of contaminating cells. However, the possibility that CsH might affect remaining contaminating cells, which in turn influence the inhibitory effect of CsH on mediator

release from human basophils, cannot be completely excluded.

In conclusion, we provide evidence that CsH is a novel, potent, and selective competitive antagonist of the FMLP receptor in human basophils. We have previously demonstrated that CsA exerts antiinflammatory activity in vitro and in vivo on the IgE-mediated activation of basophils^{35, 36} and mast cells,⁴⁵ presumably by binding to cyclophilin.^{36, 45} Thus different cyclosporins, CsA and CsH, can exert selective antiinflammatory effects on human Fc ϵ RI⁺ cells by at least two different mechanisms involving the interaction with cyclophilin and FMLP receptors, respectively. Finally, our findings are at variance with the notion that CsH is immunologically inactive and suggest that the antiinflammatory activities of cyclosporins might be more complex than originally thought. The identification of this novel property of CsH indicates how the simple isomerization of an amino acid at position 11 can markedly modify the antiinflammatory profile of cyclosporins. This suggests that single modifications in the structure of cyclosporins might lead to the identification of new compounds with selective and potent antiinflammatory effects.

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