# Demonstration of a Specific Neutrophil Receptor for a Cell-Derived Chemotactic Factor

ISAIAS SPILBERG and JAGDISH MEHTA, Department of Medicine, Washington University School of Medicine, and John Cochran Veterans Administration Hospital, St. Louis, Missouri 63106

A B S T R A C T The crystal-induced chemotactic factor, a cell-derived chemoattractant for neutrophils, binds specifically to a site on human neutrophils but not to erythrocytes or lymphocytes, suggesting a relationship between the presence of specific binding sites on the neutrophils and the ability to be chemotactically activated. The Scatchard analysis revealed an equilibrium dissociation constant at 37°C of 0.446  $\mu$ M and the presence of  $\approx 6.44 \times 10^5$  binding sites for <sup>125</sup>I-crystalinduced chemotactic factor per cell. Binding was not displaced by the synthetic chemotactic factors F-Met-Leu-Phe and Gly-His-Gly or by complement-activated plasma providing evidence of the specificity of the receptor.

# INTRODUCTION

Chemotaxis, phagocytosis, and lysosomal enzyme release are recognized functions of the neutrophil in the inflammatory response. Chemotaxis can be defined as the ability of motile cells to recognize and respond to a suitable gradient with directional migration. Presumably, the first step in the molecular mechanism of chemotactic activation includes the binding of a chemotactic factor to cell receptors. Specific binding of the synthetic chemotactic N-formylmethionyl peptides to rabbit (1) and human (2) polymorphonuclear leukocytes has been described. Although the N-formylmethionyl peptides resemble chemotactic factors produced by bacteria, specific binding to neutrophils by a human cell-derived chemotactic factor has not been demonstrated. The neutrophil-derived crystal-induced chemotactic factor (CCF),<sup>1</sup> a glycoprotein with an  $\approx 8,400$  mol wt, is thought to play an important role in the development of acute inflammation induced by monosodium urate and calcium pyrophosphate crystals

(3-5). The present study demonstrates specific binding of CCF to receptors on the human neutrophil.

### METHODS

The CCF was isolated from the lysosomal fraction of human peripheral blood neutrophils allowed to phagocytose monosodium urate crystals. The purification method, previously described (5), uses differential centrifugation and Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N. J.) chromatography followed by preparative polyacrylamide-gel electrophoresis. Human neutrophils and lymphocytes were obtained from healthy donors' blood by a Ficoll-Hypaque (Pharmacia) centrifugation technique followed by dextran sedimentation (6). The final cell preparations consisted of 95% neutrophils and 92% lymphocytes, respectively, the contaminating cells being mainly erythrocytes. Before binding studies, the cell preparations were exposed to 0.85% ammonium chloride for 5 min to lyse the contaminating erythrocytes, washed, and resuspended in the incubation medium (Hanks' solution, pH 7.4, containing 0.1% bovine serum albumin) (7) at various concentrations. Iodinated CCF was obtained using the solid phase lactoperoxidase method (8). CCF contains two histidine and one tyrosine residue (unpublished data). Immobilized lactoperoxidase beads (150  $\mu$ l suspension) obtained from Worthington Biochemical (Freehold, N. J.) were washed three times with phosphate-buffered saline (PBS), pH 7; 50  $\mu$ g of CCF in 100  $\mu$ l PBS was then added together with 0.25 to 0.5 mCi 125I-Na (Amersham Corp., Arlington Heights, Ill.) dissolved in 0.5 M phosphate buffer, pH 7.4. After gentle mixing at room temperature, the iodination reaction was initiated and continued by the addition of 25  $\mu$ l of a 1/10,000 dilution of 30%  $H_2O_2$  every 7.5 min for 30 min. Cysteine (70  $\mu$ g in 50  $\mu$ l PBS) was then added to stop the reaction. The iodinated CCF was isolated from unreactive iodine by passing the mixture through a Sephadex G-25 column  $(1.2 \times 45 \text{ cm})$ , eluted with PBS (pH 7), and stored at 4°C until used. The specific activity of the iodinated material at the time the experiments were performed appears in the legends of the pertinent figures. Greater than 95% of the radioactivity in the CCF eluate was found to be precipitable with acetone. The labeled material retained its ability to attract neutrophils, as measured by the <sup>51</sup>Cr cell-labeled technique (5), and migrated the same distance (2.5-2.7 cm) from the cathode on polyacrylamidegel electrophoresis as did the unlabeled material (5).

The binding assay was performed as follows: various concentrations of <sup>125</sup>I-CCF and cells were incubated in 0.5 ml of incubation medium for 60 min (unless specified), at 37°C with gentle agitation. Incubation was terminated by adding 0.5 ml

Received for publication 5 January 1978 and in revised form 20 <u>Sep</u>tember 1978.

<sup>&</sup>lt;sup>1</sup>Abbreviations used in this paper: CCF, crystal-induced chemotactic factor; PBS, phosphate-buffered saline.

of ice-cold incubation medium, followed by centrifugation in a Microfuge (model B, Beckman Instruments Inc., Palo Alto, Calif.) at 8,000 g for 2 min. The supernate was discarded, the pellet resuspended and transferred to another tube, and pelleted again, the supernate removed and the pellet counted for radioactivity in a gamma counter (Isomatic model 707, Baird, Atomic, Inc., Cambridge, Mass.) at 50% efficiency.

The labeled CCF preparation was subjected to binding assays in the presence of excess receptor sites to determine the proportion of radioactivity which represents "active" CCF with the capacity of interact with specific receptor sites. With a single incubation procedure, the maximum neutrophil binding activity of the labeled CCF preparation was found to be 29% of the total radioactivity of the tracer preparation (Fig. 1). The remaining radioactivity present in the media, after removal of the cells  $(1 \times 10^7 \text{ to } 4 \times 10^8)$ , although still precipitable with acetone, showed a linear decrease in chemotactic activity for neutrophils as measured by the <sup>51</sup>Cr-radiolabeled technique. At maximum binding there was no chemotactic activity detected in the supernate (Fig. 2). Likewise, the media of <sup>125</sup>I-CCF incubated with  $4 \times 10^8$  cells for 60 min showed no remaining binding activity when incubated again with new cells  $(2 \times 10^8)$ . In a separate experiment, a multiple incubation procedure was performed. A small amount of tracer (0.25  $\mu$ M) was incubated for 15 min with 2.7 × 10<sup>8</sup> cells, the supernate removed and incubated with  $2.7 \times 10^8$  cells for 45 min. The supernate was again removed for a final incubation with  $1.8 \times 10^8$  cells for 45 min. The maximum binding activity of the labeled preparation was 30.6% of the total radioactivity with this procedure. A correction factor was therefore used during calculations of receptor-binding constants (9). The radioactivity bound to the cells, after incubation with <sup>125</sup>I-CCF, was termed total binding. Nonspecific binding was defined as the amount of binding not replaced by unlabeled CCF (12 nmol), and it was  $\approx 10\%$  of the total binding (Fig. 3). Specific binding was defined as the difference between total





FIGURE 2 Relationship of <sup>125</sup>I-CCF specific binding to chemotactic activity. <sup>125</sup>I-CCF 0.147  $\mu$ M, (0.62  $\mu$ g in 0.5 ml) was incubated at 37°C for 1 h in 0.5 ml of media with 1 × 10<sup>7</sup>, 1.5 × 10<sup>8</sup>, or 4 × 10<sup>8</sup> cells; each condition was duplicated in 15 separate tubes so the total amount of tracer tested for each point was 9.3  $\mu$ g. After incubation (60 min) the cell-bound radioactivity was measured, and the supernates of duplicated tubes (representing unbound material) were pooled, dialyzed, lyophilized, reconstituted to a 1-ml final volume in Hanks'bovine serum albumin and tested for remaining chemotactic activity (5). The control <sup>125</sup>I-CCF preparation (not incubated with cells) gave a chemotactic index of four. The specific activity of <sup>125</sup>I-CCF was 36.8  $\mu$ Ci/ $\mu$ mol.

and nonspecific binding. Results are expressed as specific binding.

In a separate experiment, cells were incubated with <sup>125</sup>I-CCF for 60 min. After washing, the cells were treated with trypsin (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 0.01% in Hanks' solution at 37°C for 10 or 60 min, washed and assayed for bound radioactivity. The chemo-



FIGURE 1 Specific binding of <sup>125</sup>I-CCF by increasing concentrations of human neutrophils. The maximal receptor binding activity of the labeled chemotactic factor is 29% of the total radioactivity. <sup>125</sup>I-CCF, at a concentration of 0.25  $\mu$ M, (1.05  $\mu$ g in 0.5 ml) was added to each tube containing increasing concentrations of cells. Values represent the mean of two experiments. The range of the duplicates in each experiment was  $\pm 1.2\%$ . The specific activity of <sup>125</sup>I-CCF was 22  $\mu$ Ci/ $\mu$ mol.

FIGURE 3 Binding-competition curve for <sup>125</sup>I-labeled CCF and unlabeled CCF. Neutrophils,  $5 \times 10^6$ , were incubated for 60 min at 37°C with a mixture of 0.25  $\mu$ M <sup>125</sup>I-CCF and increasing amounts of unlabeled CCF. Values represent the mean of two experiments, each performed in duplicate. The specific activity of <sup>125</sup>I-CCF was 39  $\mu$ Ci/ $\mu$ mol.

tactic activity was evaluated by a radioassay that utilizes <sup>51</sup>Crlabeled human neutrophils as migrating cells (5).

The synthetic chemotactic factor glycyl-histidyl-glycine (Gly-His-Gly) (10) was purchased from Sigma Chemical Co. Complement-activated plasma was prepared as previously described (5). Formyl methionyl-leucyl-phenylalanine (F-Met-Leu-Phe) was a generous gift of Dr. Richard J. Freer, Medical College of Virginia, Richmond, Va.

# RESULTS

Kinetics of CCF binding. The specific binding of <sup>125</sup>I-CCF (0.25  $\mu$ M) to human neutrophils as a function of time at 37°C is shown in Fig. 4. The binding was rapid with 50% of total binding observed at <10 min, with equilibrium levels observed at 60–90 min. The binding of <sup>125</sup>I-CCF to the neutrophils was found to be reversible when nonlabeled CCF (24  $\mu$ M) was added to the mixture (Fig. 4).

Scatchard analysis of specific <sup>125</sup>I-CCF binding revealed the presence of binding sites with a dissociation constant of 0.446  $\mu$ M (Fig. 5). At saturation point, 5.35 pmol of <sup>125</sup>I-CCF was bound to the 5 × 10<sup>6</sup> neutrophils present in the incubation medium. Specific binding was linear with cell concentration over the range of  $0-2 \times 10^8$  cells.

To investigate the possibility that the observed "binding" might be due to internalization of <sup>125</sup>I-CCF, the ability of cells killed by repeated freezing and thawing to bind <sup>125</sup>I-CCF was compared to the binding observed with normally prepared cells. No difference in <sup>125</sup>I-CCF bound to disrupted or viable cells at 60 min was noticed, the disrupted cells showing 92% of the specific binding observed with viable cells. The incubation of trypsin with the cells previously incubated with <sup>125</sup>I-CCF resulted in a reduction of cell-bound



FIGURE 4 Time-course of <sup>125</sup>I-CCF binding to neutrophils. <sup>125</sup>I-CCF (0.25  $\mu$ M) was incubated with human neutrophils (1 × 10<sup>7</sup>) at 37°C for the indicated time intervals and specific binding calculated ( $\bullet$ ). To some incubation mixtures unlabeled CCF in large excess (24  $\mu$ M) was added after 30 min of incubation and <sup>125</sup>I-CCF binding assayed ( $\bigcirc$ ) at subsequent time intervals indicated. Values represent the mean of two experiments, each performed in duplicate. The specific activity of the <sup>125</sup>I-CCF preparation used was 39  $\mu$ Ci/ $\mu$ mol.



FIGURE 5 Scatchard plot of receptor-<sup>125</sup>I-CCF binding. Scatchard plot (bound:free vs. bound) of specific binding of <sup>125</sup>I-CCF to human neutrophils ( $5 \times 10^{6}$ ) at  $37^{\circ}$ C for 60 min as a function of the concentration of labeled ligand (60 nM to 2.85  $\mu$ M). The association and dissociation constants derived from the slope of the line are 2.24  $\times 10^{6}$  M<sup>-1</sup> and 0.446  $\mu$ M. Values represent the mean of three experiments, each performed in duplicate.

radioactivity. The specific binding of <sup>125</sup>I-CCF was reduced by 76% at 10 min and by 85% at 60 min after trypsin treatment.

Specificity of binding. The ability of other chemotactic factors to compete with <sup>125</sup>I-CCF for binding sites was investigated. Three apparently different chemotactic factors: the synthetic peptides F-Met-Leu-Phe and Gly-His-Gly, and complement-activated plasma were tested for their ability to inhibit CCF binding. Neither F-Met-Leu-Phe (10  $\mu$ M-10 pM), Gly-His-Gly (37  $\mu$ M-3.7  $\mu$ M), nor complement-activated plasma (10-50  $\mu$ l) altered the binding of <sup>125</sup>I-CCF to human neutrophils in our experimental design (data not shown).

In a separate set of experiments, the binding of <sup>125</sup>I-CCF to human erythrocytes  $(1 \times 10^7 - 1 \times 10^8)$  and to human peripheral blood lymphocytes  $(1 \times 10^7 - 1 \times 10^8)$  was investigated. Less than 7% of the binding observed with neutrophils was noticed with either of these cell preparations.

# DISCUSSION

Experiments performed in vitro and in vivo strongly suggest a major role for CCF in the development of the acute inflammatory response to monosodium urate crystals and possibly to other crystals as well (3, 4). The current study presents evidence that the first step in the chemotactic activation of the neutrophil by CCF involves the recognition and binding of CCF to the neutrophils by a specific receptor. Inasmuch as the amount of <sup>125</sup>I-CCF bound to intact viable cells and to freeze-thawed cells was essentially identical, and trypsin markedly displaced cell-bound radioactivity, it is likely that the binding observed was not due to endocytosis. Binding to the cells occurred rapidly, with 50% of the total specific binding observed at <10 min under experimental conditions. Assuming an equimolar ligand-receptor relationship, the number of receptors per cell based on the Scatchard plot was calculated to be  $\approx 6.44 \times 10^5$ , a number similar to the one described by Aswanikumar et al. (1) for the synthetic chemotactic factor formyl Norleu-Leu-Phe. Current data suggest that chemotactic factors induce cell response by interacting with a receptor (11). If we are indeed measuring a specific receptor responsible for mediating chemotaxis in human neutrophils, a close correlation should exist between the  $K_d$  of the receptor and the concentration of the chemotactic factor required to give half-maximal chemotactic response (12, 13). The Scatchard analysis of specific <sup>125</sup>I-CCF binding revealed the presence of a binding site with a dissociation constant of 0.446  $\mu$ M a value close to the concentration of CCF required to elicit a half-maximal chemotactic response,  $1.5 \ \mu M$  (5). A relationship of a similar degree has been reported by Aswanikumar et al. (1) for the synthetic factor formyl Norleu-Leu-Phe and its rabbit neutrophil receptor.

Human erythrocytes and peripheral blood lymphocytes showed no significant binding of <sup>125</sup>I-CCF. Lymphocytes, although known to respond to a suitable chemotactic challenge (8), do not respond chemotactically to a gradient of CCF (unpublished observation) and human erythrocytes are known to be nonmotile cells. These results suggest a relationship between the presence of specific binding sites on the cell and the ability of a given cell to be chemotactically activated by CCF.

No decrease in binding of <sup>125</sup>I-CCF to neutrophils was observed when complement-activated serum or the synthetic chemotactic peptides Gly-His-Gly and F-Met-Leu-Phe were tested in the competition studies. The maximal concentrations of the synthetic chemoattractants tested were well above those required to elicit half-maximal chemotactic response from the human neutrophil (2, 10). The main active factor in complement activated plasma, C5a (14), has been described in similar experiments not to compete for binding sites with the formyl peptides (1, 2). These results are in favor of the existence of distinct specific receptors for chemotactic factors in the neutrophil. However, the possibility that receptors may not be required for some chemotactic factor(s) to elicit a chemotactic response in the neutrophil cannot be ruled out.

In conclusion, the cell-derived chemotactic factor CCF binds to human neutrophils in a saturable, timedependent fashion with a calculated dissociation constant of 0.446  $\mu$ M. Binding of the ligand to viable and disrupted cells is alike indicating that binding is not accounted for by endocytosis. The binding is not displaced by the chemotactic substances Gly-His-Gly, F-Met-Leu-Phe, or complement-activated plasma providing evidence for the specificity of the receptor.

## ACKNOWLEDGMENTS

We wish to thank Mr. Steven Disper and Mrs. Vanita Venugopal for their technical assistance, and Ms. Gerlean Smith for secretarial help.

This investigation was supported by U. S. Public Health Service grant AM-19349 and by an Arthritis Foundation Clinical Research Center grant.

### REFERENCES

- Aswanikumar, S., B. Corcoran, E. Schiffman, A. R. Day, R. J. Freer, H. J. Showell, E. L. Becker, and C. B. Pert. 1977. Demonstration of a receptor on rabbit neutrophils for chemotactic peptides. *Biochem. Biophys. Res. Commun.* 74: 810-817.
- Williams, L. T., R. Snyderman, M. Rike, and R. J. Lefkowitz. 1977. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* 74: 1204-1208.
- 3. Spilberg, I., D. Rosenberg, and B. Mandell. 1977. Induction of arthritis by purified cell derived chemotactic factor. J. Clin. Invest. 59: 582-585.
- 4. Spilberg, I., A. Gallacher, and B. Mandell. 1977. Calcium pyrophosphate dihydrate (CPPD) crystal induced chemotactic factor: subcellular localization, role of protein synthesis and phagocytosis. J. Lab. Clin. Med. 89: 817– 822.
- 5. Spilberg, I., A. Gallacher, J. Mehta, and B. Mandell. 1976. Urate crystal induced chemotactic factor. Isolation and partial characterization. J. Clin. Invest. 58: 815-819.
- 6. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21(Suppl. 97): 77-89.
- Ward, P. A., E. R. Unanue, S. J. Goralnick, and G. F. Schneider. 1977. Chemotaxis of rat lymphocytes. J. Immunol. 119: 416-421.
- 8. David, G. S., and R. A. Reisfeld. 1974. Protein iodination with solid state lactoperoxidase. *Biochemistry*. 13: 1014-1021.
- 9. Catt, K. J., J-M. Ketelsiegers, and M. L. Dufau. 1976. Receptors for gonadotropic hormones. *In* Methods in Receptor Research. Pt. 1. Melvin Blecher, editor. Marcell Dekker, Inc., New York and Basel. 175–250.
- Spilberg, I., B. Mandell, J. Mehta, T. Sullivan, and L. Simchowitz. 1978. Dissociation of the neutrophils functions of exocytosis and chemotaxis. J. Lab. Clin. Med. 92: 297-302.
- 11. Zigmond, S. H. 1978. Chemotaxis by polymorphonuclear leukocytes. J. Cell Biol. 77: 269-287.
- 12. Mesibov, R., G. W. Ordal, and J. Adler. 1973. The range of attractant concentrations for bacterial chemotaxis and the threshold and size of response over this range. J. Gen. Physiol. 62: 203-223.
- 13. Zigmond, S. H. 1977. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. J. Cell Biol. 75: 606-616.
- Becker, E. L., H. J. Showell, P. M. Henson, and L. S. Hsu. 1974. The ability of chemotactic factors to induce lysosomal enzyme release. I. The characteristics of the release, the importance of surfaces and the relation of enzyme release to chemotactic responsiveness. J. Immunol. 112: 2047-2054.