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Research Article

Monocytes, macrophages, and neutrophils will demonstrate several important cellular functions in response to synthetic formylated oligopeptides. N-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine (fNLPNTL) was a potent chemoattractant for human blood monocytes; a 1.0-nM concentration induced a maximal chemotactic response. Binding of 125I-labeled fNLPNTL to the monocyte formyl peptide receptor was rapid, specific, and saturable at 4, 24, or 37 degrees C. At 4 degrees C, monocytes from several different donors demonstrated between 10,000 and 18,000 receptors/cell with a dissociation constant (Kd) of 1.7-2.7 nM. The association of the 125I peptide with the cells was irreversible at the elevated temperatures and exceeded the amount of surface receptor by approximately four-fold, suggesting receptor-mediated peptide endocytosis. Processing of rhodamine-labeled fNLPNTL by monocytes was observed directly by video intensification microscopy. At 37 degrees C, diffuse membrane fluorescence was seen initially, followed by rapid aggregation and internalization of the peptide. Monocytes incubated with fNLPNTL displayed a temperature dependent loss of surface binding capacity (receptor down-regulation). This decrease was due to a decrease in surface receptor number rather than to a decrease in receptor affinity. A dose-response curve for peptide-induced receptor down-regulation correlated with a dose-response curve for 125I-labeled fNLPNTL uptake, suggesting that each uptake event led to the loss of one surface receptor. Surface receptor replenishment following down-regulation was rapid and not dependent on new protein synthesis, but was inversely […]

Monocyte Chemotactic Peptide Receptor

FUNCTIONAL CHARACTERISTICS AND LIGAND-INDUCED REGULATION

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ABSTRACT Monocytes, macrophages, and neutrophils will demonstrate several important cellular functions in response to synthetic formylated oligopeptides. *N*-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine (fNLPNTL) was a potent chemoattractant for human blood monocytes; a 1.0-nM concentration induced a maximal chemotactic response. Binding of ^{125}I-labeled fNLPNTL to the monocyte formyl peptide receptor was rapid, specific, and saturable at 4, 24, or 37°C. At 4°C, monocytes from several different donors demonstrated between 10,000 and 18,000 receptors/cell with a dissociation constant (K_d) of 1.7–2.7 nM. The association of the ^{125}I peptide with the cells was irreversible at the elevated temperatures and exceeded the amount of surface receptor by approximately fourfold, suggesting receptor-mediated peptide endocytosis. Processing of rhodamine-labeled fNLPNTL by monocytes was observed directly by video intensification microscopy. At 37°C, diffuse membrane fluorescence was seen initially, followed by rapid aggregation and internalization of the peptide.

Monocytes incubated with fNLPNTL displayed a temperature dependent loss of surface binding capacity (receptor down-regulation). This decrease was due to a decrease in surface receptor number rather than to a decrease in receptor affinity. A dose-response curve for peptide-induced receptor down-regulation correlated with a dose-response curve for ^{125}I-labeled fNLPNTL uptake, suggesting that each uptake event led to the loss of one surface receptor. Surface receptor replenishment following down-regulation was rapid and not dependent on new protein synthesis, but was inversely related to both the time and peptide concentration used to induce down-regulation. An exact correlation between receptor down-regulation and functional deactivation of the chemotactic response could not be demonstrated.

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INTRODUCTION

Cells of the mononuclear phagocyte system (blood monocytes and tissue macrophages) play a major role in various physiologic and pathologic conditions including removal and catabolism of senescent or damaged erythrocytes (1), "processing" of antigen for initiation of immune responses (2), participation in inflammatory processes (3), promotion of wound healing (4), and mediation of host defense against microbes (5) and neoplastic cells (6). Although all of these important cellular functions presumably are dependent on the ability of the mononuclear phagocyte to recognize various environmental stimuli at the plasma membrane, little is known about the molecular mechanisms of these recognition processes.

The movement of monocytes or macrophages to various tissue sites and sites of inflammation in vivo is thought to be influenced by various chemotactic substances including C5a, lymphokines, and bacterial-derived factors (7, 8). Neutrophil membrane receptors for molecularly defined N-formylated oligopeptides, which are believed to be synthetic analogues of the bacterial-derived factors, have been demonstrated, and factors modulating neutrophil chemotactic peptide binding have been studied (9–14). In this investigation, we characterize the receptor for the iodinated chemotactic peptide *N*-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine (fNLPNTL)[1] on human blood monocytes, study peptide-induced modulation of this receptor, and attempt to correlate receptor modulation with functional deactivation of the chemotactic response.

METHODS

Materials. Dulbecco's modified Eagle's medium (DMM, catalog 430-1600, Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) was formulated with endotoxin-free water and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM Hepes, 1 mg/ml dextrose, and sterilized by filtration. The Limulus amebocyte lysate assay confirmed that this media was free of endotoxin (15, 16). Ficoll-sodium diatrizoate, specific gravity 1.077 (Histopaque-1077) was from Sigma Chemical Co., St. Louis, Mo., and Gey's balanced salt solution with 2% bovine serum albumin (Gey's-BSA) was from Flow Laboratories, McLean, Va. Assay wash buffer was 15 mM phosphate-buffered isotonic saline (PBS) with 1 mM $CaCl_2$ and 0.1% BSA. PBS was buffered with dibasic sodium phosphate and monobasic potassium phosphate (67 mM). 96-well microtiter plates (Microtest II, number 3040) were from Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif. A semi-automated multiple sample harvester (Otto Hiller Co., Madison, Wis.) was used with glass fiber filters (Whatman 5679, grade 943AH, Whatman Inc., Clifton, N. J.). Non-specific esterase (alpha naphthyl butyrate esterase) stains were done with reagents from Technicon Instruments Corp., Tarrytown, N. Y. (17), and peroxidase stains were done by the method of Kaplow (18). Rabbit IgG anti-sheep erythrocyte was from Cordis Laboratories, Inc. (Miami, Fla.). Sheep erythrocytes were opsonized as previously described (19). Polystyrene latex spheres (1.01 μm Diam) were from Difco Laboratories, Detroit, Mich. Polyvinylpyrrolidone-coated silica (Percoll) was from Pharmacia Fine Chemicals (Uppsala, Sweden). Tritiated leucine (5 Ci/mmol sp act) was from New England Nuclear (Boston, Mass.). Leucine-free minimal essential medium (MEM, catalog number 320-1895) was from Gibco Laboratories.

fNLPNTL and *N*-formyl-methionyl-leucyl-phenylalanyl-methionyl-tyrosine (fMLPMT) were synthesized as previously described (12). fNLPNTL was radioiodinated by the chloramine T method (12) with specific radioactivities varying from 450 to 1,700 Ci/mmol among various samples. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was obtained from Sigma Chemical Co. The peptides were prepared as 1-mM solutions in dimethylformamide (fNLPNTL) or dimethyl sulfoxide (fMLP or fMLPMT) and diluted in Gey's-BSA to 1 μM (unlabeled peptides) or 20 nM (labeled peptides) and frozen in aliquots at −20°C until use.

Isolation of monocytes. We obtained fresh peripheral blood monocytes from normal volunteers or patients after their informed consent according to a protocol approved by the Duke University Clinical Investigation Committee. The blood, anticoagulated with 10 U/ml heparin, was diluted 1:3 with PBS and layered over Ficoll-sodium diatrizoate, and the mononuclear cells were separated according to methods of Boyum (20), with the modification of slow spins (60 *g*) during the washing steps to decrease platelet contamination. After suspending the resulting mononuclear cells at 4×10^6/ml DMM, 0.1 ml was placed into 6-mm Diam chambers, and incubated for 2 h at 37°C in 5% CO_2/95% air to allow for monocyte adherence. The nonadherent cells were removed by washing the chambers twice with 0.2 ml PBS using suction through a Pasteur pipette. Usually, 70% of the adherent cells were esterase- and peroxidase-positive, ingested opsonized erythrocytes and polystyrene spheres, and were contaminated by 30% lymphocytes; also, two to four platelets per leukocyte remained after washing. Platelets and lymphocytes do not bind the chemotactic peptide (21).

To more completely isolate monocytes from lymphocytes, the mononuclear cells obtained after processing with Ficoll-sodium diatrizoate were separated using a discontinuous Percoll gradient with a modification of a previously described technique (22). 50×10^6 monocytes obtained from Ficoll-sodium diatrizoate gradients were incubated 20 min at 37°C with 2 nM ^{125}I-labeled fNLPNTL (total) or 2 nM ^{125}I-labeled fNLPNTL with 100 nM unlabeled fNLPNTL (nonsaturable), and washed three times with DMM. Then the cells, in a 0.5-ml PBS in a 15-ml centrifuge tube, were overlaid sequentially with 4 ml Percoll at specific gravity 1.126, 4 ml at specific gravity 1.067, and 2 ml at specific gravity 1.057. The tube was centrifuged at 420 *g* for 20 min at 4°C. Cells from the resulting bands were washed three times with PBS. The cell-associated ^{125}I-labeled fNLPNTL was determined and specific peptide uptake was expressed as the difference between the total and nonsaturable values.

Binding assay. The standard binding assay was done in microtiter plates. The adherent cell layer was washed with PBS, and then 0.15 ml of the radiolabeled peptide (2 nM in Gey's-BSA unless otherwise noted) was placed directly onto the adherent cells. Replicate wells received mixtures of the radiolabeled peptide and saturating (100 nM) concentrations of the unlabeled peptide, so that nonsaturable binding could be determined for each experimental point. All assays were done in triplicate. The cells were incubated with the peptide for 2 h at the designated temperature, and then the adherent cells were aspirated from the microtiter chambers with a semiautomated multiple sample harvester, deposited onto filter paper, and washed with 20 vol of assay wash buffer. The filters were then counted in a gamma counter to determine cell-associated radioactivity. All data are presented as saturable binding, the difference between total and nonsaturable binding. Giemsa-stained cells in replicate wells were counted and binding was expressed as femtomoles bound per 10^5 adherent cells. Careful, uniform washing was necessary to lessen the variability in cell number per replicate chamber. In studies reported here, standard deviations of cell numbers in replicate chambers were <15% of the mean. In down-regulation experiments, the cells were incubated in suspension with the designated concentration of unlabeled fNLPNTL in protein-free DMM at 5×10^6 cells/ml. They were then washed three times with cold DMM and used for subsequent recovery and binding assays. The binding assay was done in suspension with the cells at 5×10^6/ml in 50% DMM/50% assay wash buffer with 0.1% BSA (12).

Visualization of binding and uptake of the tetramethylrhodamine conjugate of fNLPNTL was done using video intensification microscopy as previously described (21). Photographs were taken with a Polaroid camera (Polaroid Corp., Cambridge, Mass.) directly from the television monitor. Chemotaxis assays were done as previously described using polycarbonate filters with pores of 5 μM Diam (23). Measurement of leucine incorporation into trichloroacetic acid-precipitable monocyte material was done as described previously (24).

RESULTS

Characteristics of ^{125}I-formyl peptide binding and uptake by human monocytes. The synthetic oligo-

[1] *Abbreviations used in this paper*: DMM, supplemented Dulbecco's modified Eagle's medium; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; fMLPMT, *N*-formyl-methionyl-leucyl-phenylalanyl-methionyl-tyrosine; fNLPNTL, *N*-formyl-norleucyl-leucyl-phenylalanyl-tyrosyl-lysine; Gey's-BSA, Gey's balanced salt solution with 2% bovine serum albumin; PBS, phosphate-buffered saline.

peptide fNLPNTL is a potent chemoattractant for human neutrophils and differentiated human promyelocytic leukemia cells (HL-60) (12, 25). Both cell types exhibit a maximal chemotactic response to a 1.0 nM concentration. fNLPNTL was also a potent chemoattractant for human monocytes, with a 1.0-nM concentration again inducing the maximal response (Fig. 1).

When human monocytes, either adherent to plastic or in suspension, were incubated with 2 nM ^{125}I-labeled fNLPNTL, cell-associated radioactivity increased until a stable plateau was achieved (Fig. 2). At 24 and 37°C, this plateau was reached in <1 h, whereas at 4°C, both the rate and extent of accumulation of cell-associated radioactivity was less. The plateau at 4°C was a true equilibrium, because cell-associated radioactivity could be removed by washing, whereas the plateau at 24°C was not, because the cell-associated radioactivity could not be displaced by washing or by the addition of a 50-fold excess of nonradioactive formyl peptide (Fig. 3). The plateau at 24°C was not due simply to proteolytic destruction of ^{125}I-labeled fNLPNTL in solution, as had been demonstrated under similar incubation conditions with human neutrophils (12). The cell-free supernates obtained from a 2- or 30-min binding incubation at 24°C supplied enough native ^{125}I-labeled fNLPNTL to yield normal uptake values when applied to fresh cells at 24°C.

Both cell surface peptide binding at 4°C and receptor-mediated peptide uptake at 24°C were saturable with increasing peptide concentration; a half-maximal value was obtained at 2–3 nM, with a plateau >10 nM (Fig. 4). At saturation, uptake of 8 fmol/10^5 cells at 24°C corresponded to ~50,000 uptake sites per cell. This value was fourfold greater than the 12,000 surface receptors measured at saturation at 4°C. An uptake value of 4.7±2.3 fmol of ^{125}I-labeled fNLPNTL per 10^5 cells (range 1.6 to 10 fmol) was demonstrated for adherent mononuclear cells isolated from nine different donors using 2 nM ^{125}I-fNLPNTL at 24°C. Cells from a single donor (J.J.M.) were assayed 15 times over a 4-mo period using these same assay conditions and a mean uptake of 1.9±0.8 fmol per 10^5 cells (range 0.7 to 3.5 fmol) was determined. Nonsaturable binding and uptake measured in the presence of 100 nM unlabeled fNLPNTL was 20–24% of the total cell-associated radioactivity. The potencies of unlabeled fNLPNTL, fMLP, and fMLPMT as inhibitors of ^{125}I-labeled fNLPNTL uptake (Fig. 5) correlated well with their respective potencies as chemoattractants (12). Varying the pH between 6 and 7.7, or the calcium concentration

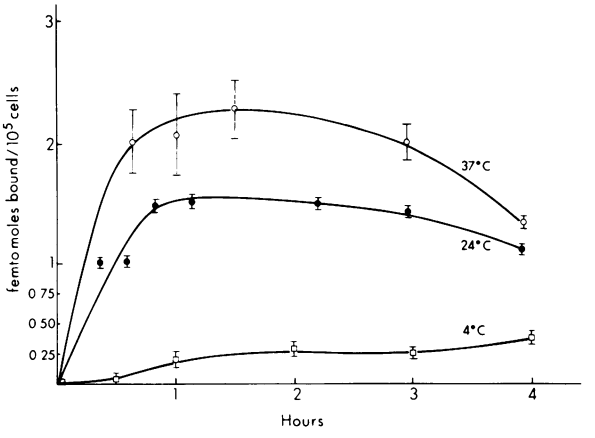

FIGURE 2 Time-course of uptake of ^{125}I-labeled fNLPNTL by human monocytes at various temperatures. The assay conditions were those described in the Methods section except the binding was done at 4 (□), 24 (●), or 37°C (○) for various timed intervals as indicated. Values represent mean ±1 SEM.

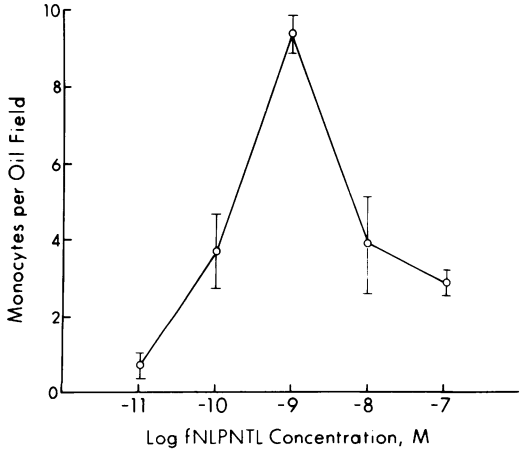

FIGURE 1 Chemotactic response of monocytes of fNLPNTL. The values represent means±1 SD.

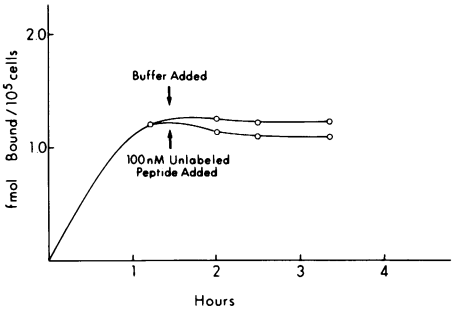

FIGURE 3 Attempted displacement of ^{125}I-labeled fNLPNTL from human monocytes by unlabeled peptide. The binding assay was done under standard conditions but at 90 min the incubation medium was aspirated and Gey's-BSA or 100 nM unlabeled fNLPNTL was added to the chambers (added volume 0.15 ml). The cells were then harvested at the designated times for determination of cell-associated labeled peptide.

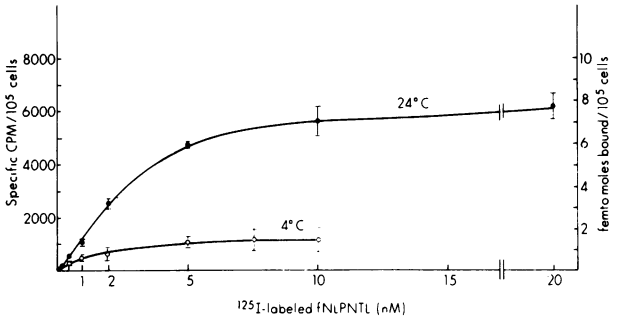

FIGURE 4 Cell-associated ^{125}I-labeled fNLPNTL as a function of peptide concentration. The assay was done as described in the Methods section with an incubation time of 2 h at 24 (●) or 4°C (○). Values represent mean±1 SEM.

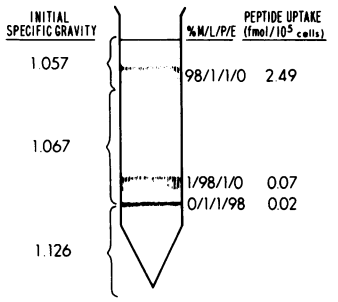

FIGURE 6 ^{125}I-labeled fNLPNTL uptake by mononuclear cells isolated from a discontinuous Percoll gradient. The percent of monocytes (M), lymphocytes (L), polymorphonuclear leukocytes (P), and erythrocytes (E) in each band was determined by Giemsa and nonspecific esterase staining.

between 0.13 and 1.50 mM did not significantly alter the uptake, although there was a slight increase at 1.00 mM calcium.

When the mononuclear cells obtained by Ficoll-Hypaque separation were further fractionated using discontinuous Percoll gradients, all of the specific ^{125}I-labeled fNLPNTL uptake was localized to the monocyte fraction (Fig. 6). Contaminating monocytes and neutrophils accounted for the small amount of uptake seen in the lymphocyte and erythrocyte fraction.

Fluorescent formyl peptide uptake. The inability to remove ^{125}I-labeled fNLPNTL that had been bound at the higher temperatures suggested that the peptide had been internalized as had been previously shown with human and rabbit neutrophils (12, 14, 21). A direct demonstration of receptor-mediated internalization was obtained using rhodamine-labeled fNLPNTL and video intensification microscopy (21).

When adherent human monocytes were incubated with rhodamine-labeled fNLPNTL at 37°C and fixed at various timed intervals, the following sequence of events was determined. At 1 min, the fluorescent peptide was distributed in a diffuse pattern over the entire cell membrane (Fig. 7A). By 3 min, the membrane-associated fluorescent peptide had begun to aggregate in the plane of the membrane, and by 15 min, it had been internalized within small endocytic vesicles that displayed the saltatory motion characteristic of cytoplasmic organelles (Fig. 7B, C). These fluorescent events were prevented in the presence of 100 nM nonfluorescent fNLPNTL, demonstrating that the events were receptor-mediated, rather than due to nonspecific membrane adsorption or of fluid-phase pinocytosis. Contaminating lymphocytes, platelets, and erythrocytes did not demonstrate saturable fluorescent peptide binding or uptake. All mononuclear cells that exhibited the ability to spread on glass always internalized the rhodamine-peptide. Occasionally, a cell with a nuclear morphology characteristic of a monocyte did not spread on the coverslip. These cells did not bind or internalize the rhodamine-peptide. Whether these cells are lymphocytes or a small subset of monocytes is unknown.

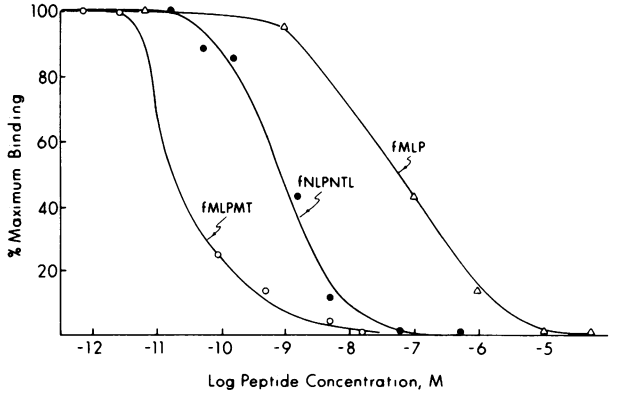

FIGURE 5 Inhibition of ^{125}I-labeled fNLPNTL uptake by human monocytes by unlabeled chemotactic peptides. The uptake was determined using the standard uptake assay with unlabeled peptide [fMLPMT (○), fNLPNTL (●), fMLP (△)] mixed with ^{125}I-labeled fNLPNTL at indicated concentrations prior to addition to the cells.

Down-regulation of receptors. Cells that had been treated with increasing concentrations of unlabeled fNLPNTL for 30 min at 37°C and subsequently washed extensively at 4°C displayed a diminished capacity to bind ^{125}I-labeled fNLPNTL at 4°C (Fig. 8). This phenomenon has been observed in many ligand-receptor systems and is termed down-regulation. Half-maximal down-regulation was induced by 2 nM formyl peptide, with a plateau >10 nM. The percentage of maximal down-regulation induced by a given concentration of unlabeled peptide paralleled the percentage of maximal uptake measured with the same concentration of ^{125}I-labeled fNLPNTL (see Fig. 4). Typically, 5–15% of the receptors were not down-regulated even after prolonged incubations with very high peptide

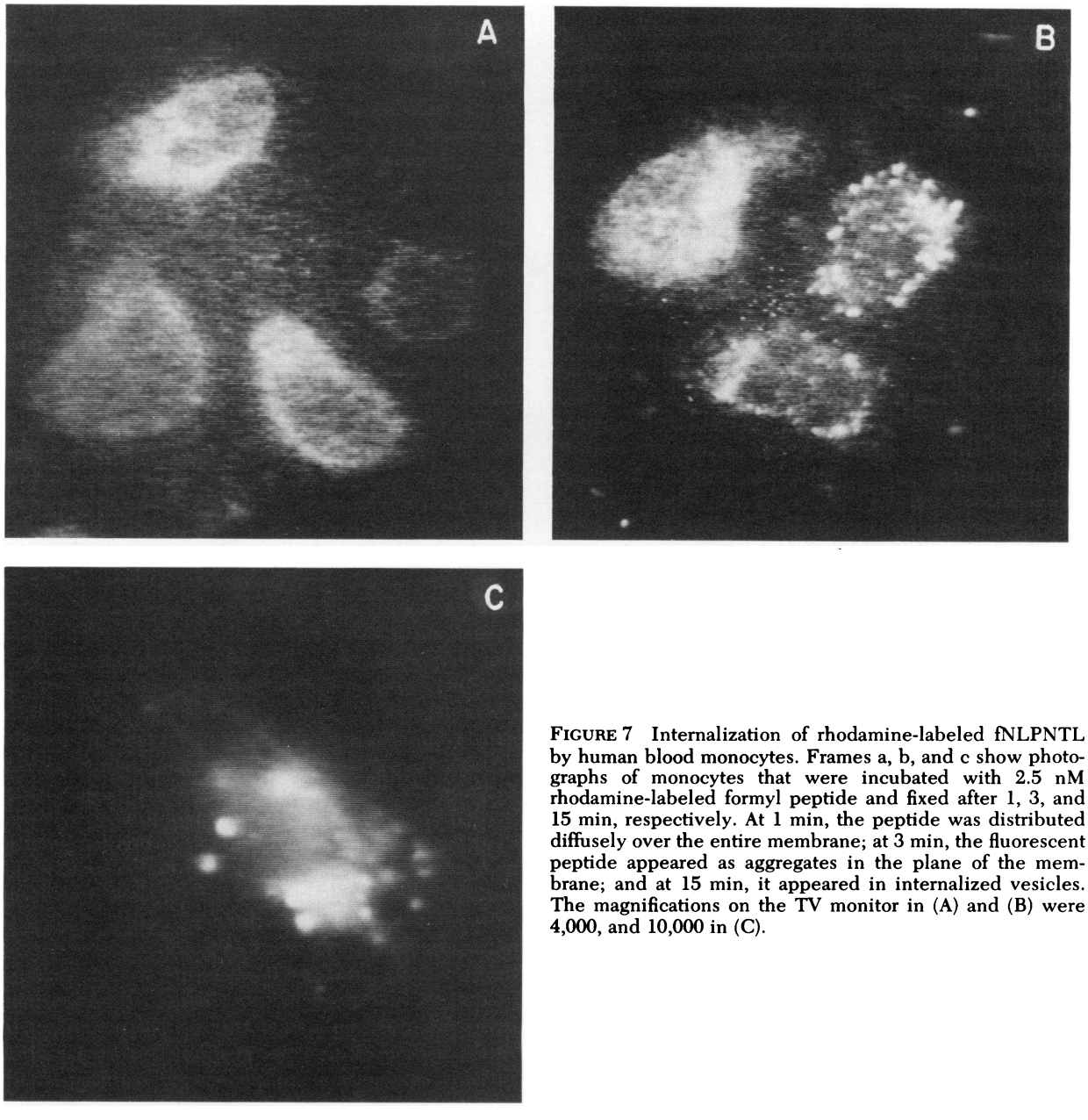

FIGURE 7 Internalization of rhodamine-labeled fNLPNTL by human blood monocytes. Frames a, b, and c show photographs of monocytes that were incubated with 2.5 nM rhodamine-labeled formyl peptide and fixed after 1, 3, and 15 min, respectively. At 1 min, the peptide was distributed diffusely over the entire membrane; at 3 min, the fluorescent peptide appeared as aggregates in the plane of the membrane; and at 15 min, it appeared in internalized vesicles. The magnifications on the TV monitor in (A) and (B) were 4,000, and 10,000 in (C).

concentrations (200–500 nM). This decrease in surface binding was temperature dependent. 11% of the surface receptors were down-regulated following a 1-h incubation at 4°C with 5 nM formyl peptide, whereas 80% of the receptors were down-regulated by 5 nM peptide within 15 min at 37°C. That the diminished binding capacity of down-regulated cells was primarily due to a decrease in functional surface receptors rather than to an altered receptor affinity is shown in Fig. 9. Scatchard analysis demonstrated that although the total binding capacity of down-regulated cells was decreased to 5% of the control value (18,000 receptors/control cell, 900 receptors/down-regulated cell), the dissociation constant (K_d) values were comparable (K_d = 1.7 nM for control cells, K_d = 1.1. nM for down-regulated cells).

Receptor recovery after down-regulation. We next attempted to correlate this receptor loss with an alteration in the chemotactic response of the down-regulated cells. Although the down-regulated cells always displayed diminished chemotaxis when compared with control cells, an exact correlation between extent of down-regulation and decrease in the chemotactic response was not found for any single experiment

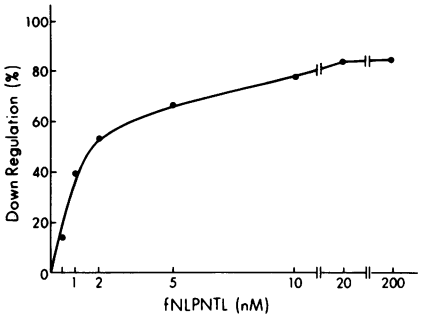

FIGURE 8 Down-regulation of ^{125}I-labeled fNLPNTL receptors on monocytes as a function of peptide concentration used to pretreat monocytes. The cells were treated 30 min at 37°C with the designated peptide concentrations and washed at 4°C. Peptide binding was then determined after a 2-h incubation with 2 nM ^{125}I-labeled fNLPNTL at 4°C.

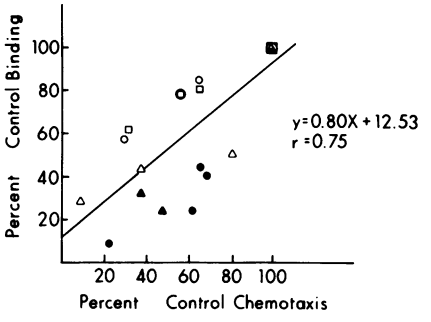

FIGURE 10 Correlation of chemotaxis and ^{125}I-labeled fNLPNTL binding in down-regulated cells. The cells were treated for 30 min at 37°C with various concentrations of fNLPNTL, washed at 4°C, and then separate aliquots were assayed for binding of 2 nM ^{125}I-labeled peptide and for chemotactic response to 2 nM fNLPNTL. Results from five separate experiments designated by the different symbols are shown. Each point represents the percentage of control binding and percentage of control chemotaxis of a single population of treated cells. The line that best fit the experimental points was drawn using the designated formula calculated by the method of least squares.

and poor reproducibility was evident between experiments. However, when the data from five experiments were combined, a correlation coefficient of $r = 0.75$ was obtained, suggesting that chemotactic receptor number may correlate with the magnitude of the chemotactic response (Fig. 10). The variability evident in these experiments could be explained if receptor recovery were occurring during the 90- to 120-min incubation at 37°C required for the chemotaxis assay, as had been shown for rabbit neutrophils (14). The rate of receptor recovery at 37°C is shown in Fig. 11 for cells down-regulated with three different peptide concentrations. Particularly at the lower peptide concentrations (near the K_d), receptor recovery was rapid and frequently exceeded control values. Typically, ~50% of the down-regulated receptors were replaced within the first 15 min of the incubation. This rapid return was then followed by a slower phase, so that complete recovery was achieved by 60–120 min. Cells treated with very high peptide concentrations either recovered slowly and incompletely, or not at all during the

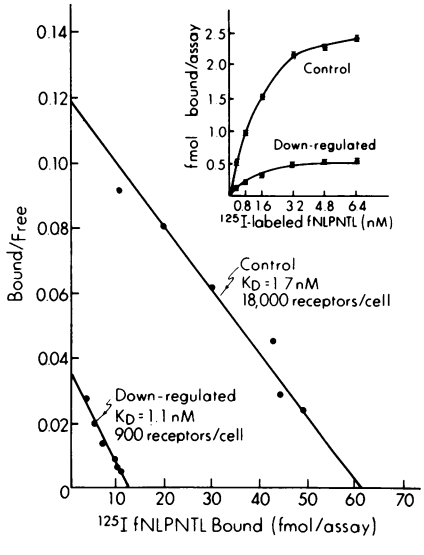

FIGURE 9 Scatchard analysis of binding of ^{125}I-labeled fNLPNTL to control and down-regulated cells. Cells were treated for 30 min at 37°C with 10 nM fNLPNTL (down-regulated) or Gey's-BSA (control) and washed three times at 4°C. Binding at the indicated concentrations of ^{125}I-labeled fNLPNTL (inset) was then determined at 4°C for 2 h with 2×10^6 cells/300 μl (control) or 8×10^6 cells/300 μl (down-regulated) and the mean values plotted ±1 SEM. These data were analyzed by the method of Scatchard and the line that best fit the points calculated by the method of least squares.

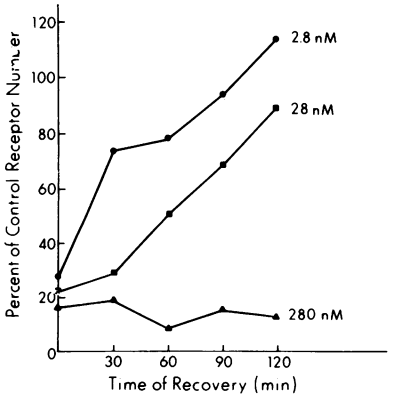

FIGURE 11 Time of monocyte receptor recovery after down-regulation with various concentrations of fNLPNTL. The cells were treated with 0, 2.8, 28, or 280 nM peptide for 30 min at 37°C, washed at 4°C, and incubated at 37°C. At the designated times, cells were assayed for binding after 2-h incubation at 4°C with 2 nM ^{125}I-labeled fNLPNTL.

2-h recovery incubation. However, when these cells were then cultured overnight, 50% receptor recovery was seen at 8 h and complete recovery at 18 h. As can be seen from this figure, an exact correlation between the extent of down-regulation measured at 4°C at zero time and the chemotactic response measured at 37°C over a 90–120-min interval would not be expected because the receptor number per cell would be increasing during the chemotaxis assay. For example, in one experiment, cells treated with 2.8 nM peptide were 72% down-regulated at zero time, but displayed rapid receptor recovery and essentially normal chemotaxis. By contrast, cells treated with 280 nM peptide were down-regulated to a similar extent (85%) at zero time, but receptor recovery did not occur and their chemotactic response was inhibited 92%.

The extent of receptor recovery after down-regulation was inversely related to the time of the incubation used to induce down-regulation (Fig. 12). The receptor number on mononuclear cells was down-regulated to ~10% of the control value by incubations of 15, 30, or 60 min at 37°C with 20 nM unlabeled fNLPNTL. However, when these cells were washed extensively and receptor recovery was assessed following a 60-min incubation at 37°C, cells down-regulated for 15 min recovered to 100% of control receptor number and cells down-regulated for 30 min recovered to 70% of control, whereas cells down-regulated to 60 min recovered to only 46% of the control value.

Receptor recovery appeared to be independent of new protein synthesis (Fig. 13). Cells were 88% down-regulated with 20 nM fNLPNTL for 30 min at 37°C, washed extensively at 4°C, and the time-course of receptor recovery was determined at 37°C in the absence or presence of 3 μg/ml cycloheximide. This concentration of cycloheximide inhibited leucine incorporation into monocyte acid-precipitable material by 89%

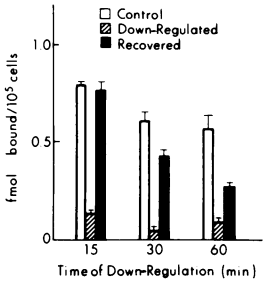

FIGURE 12 Monocyte peptide receptor recovery as a function of time of down-regulation. Monocytes were treated at 37°C with 0 (control) or 20 nM (down-regulated) fNLPNTL for 15, 30, or 60 min, washed at 4°C, and then incubated in the absence of peptide for 60 min at 37°C. Binding of 2 nM ^{125}I-labeled fNLPNTL at 4°C for 2 h was determined immediately after the pretreatment and after the 60-min recovery incubation (recovered). Error bars represent 1 SEM.

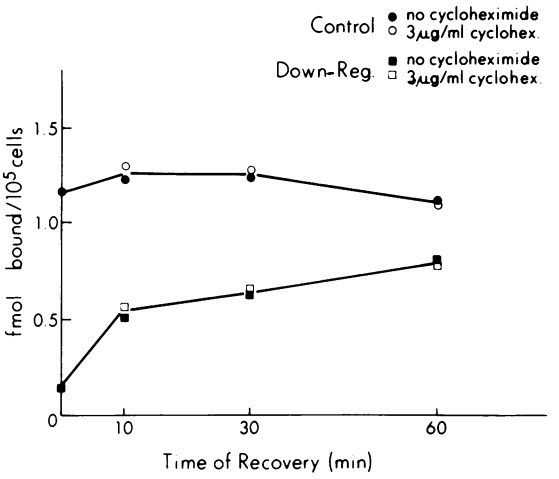

FIGURE 13 The effect of protein synthesis inhibition by cycloheximide on monocyte peptide receptor recovery after down-regulation. Monocytes were treated 30 min at 37°C with 0 (control) or 20 nM (down-regulated) fNLPNTL, washed at 4°C, and then incubated at 37°C with or without 3 μg/ml cycloheximide. Binding of 2 nM ^{125}I-labeled fNLPNTL at 4°C for 2 h was then determined following the designated times of recovery.

without affecting cell viability during a 2-h incubation. Receptor number per cell was identical at each time point in the presence or absence of cycloheximide. Although in the experiment depicted, receptor number per control cell remained essentially constant over the 60-min incubation at 37°C, in related experiments we frequently observed a 10–40% decrease in receptor number in the absence of peptide during a 60-min incubation.

DISCUSSION

As demonstrated here, the properties of the human blood monocyte receptor for chemotactic formyl peptides resemble the properties of the receptor on human and rabbit neutrophils and guinea pig macrophages (10–12, 14, 26). As determined at 4°C, there are 10,000–18,000 surface receptors per cell that mediate the rapid internalization and specifically bound ^{125}I-labeled fNLPNTL. The relative affinities of a small series of peptides correlated with their relative potencies as chemoattractants. Rhodamine-labeled fNLPNTL was initially bound in a relatively diffuse distribution over the cell surface, but the bound peptides rapidly aggregated in the plane of the plasma membrane and were internalized. Receptor-mediated binding, aggregation, and internalization appear to be universal mechanisms by which eukaryotic cells process polypeptide ligands (27).

The way in which the receptor itself is processed during peptide-binding and internalization is less

clear. The number of surface receptors decreased following cell-peptide interactions at 37°C, but not at 4°C. The dose-response curve for this peptide-induced receptor down-regulation correlated closely with the dose-response curve for peptide uptake under the same conditions (Fig. 8). This implies that for each peptide internalized, one surface receptor became unavailable for binding. A similar one-to-one relationship has been postulated for down-regulation of the formyl peptide receptor on rabbit neutrophils (14), although other investigators found that receptor down-regulation exceeded peptide uptake (28). The decrease in cell-surface binding appeared to be due to surface receptor loss rather than to a change in receptor affinity because half-maximal saturation of binding occurred at ~2 nM for both the receptors on control cells and the receptors remaining on the down-regulated cells (Fig. 9). Of course, a large decrease in affinity (perhaps two orders of magnitude or greater) would be indistinguishable from receptor loss because at the high ^{125}I-labeled peptide concentrations (>100 nM) necessary to assay this postulated low affinity receptor, nonsaturable binding would obscure any saturable binding component. As noted by others (14, 28, 29) 5–15% of the surface receptors frequently could not be down-regulated even after incubation of the cells with 200–500 nM formyl peptide. These residual receptors were indistinguishable by binding analysis from the majority of receptors that were rapidly down-regulated.

Endocytosis of the peptide-receptor complex as a unit is one possible explanation for receptor down-regulation, although a peptide-induced conformational change or other modification of the surface receptor, which would inhibit subsequent peptide binding, is also possible. We have been unable to design an experiment to distinguish receptor endocytosis from inactivation of receptors that remain on the cell surface.

The amount of peptide internalized when the uptake plateau was reached at 24 or 37°C consistently exceeded the number of surface receptors (measured at 4°C) by approximately fourfold (Figs. 2 and 4). This is not consistent with the simple hypothesis that each uptake event inactivates one surface receptor and implies that replenishment of functional surface receptors must occur. The term "replenishment" is used throughout in a functional sense, to denote the recovery of specific, saturable surface binding after receptor down-regulation, without implication as to mechanism. The data in Fig. 11 demonstrate that the receptors that were down-regulated by the binding event were subsequently replenished on the plasma membrane during a 37°C incubation. Moreover, the rate and perhaps extent of recovery appeared to depend on the peptide concentration used for induction of down-regulation. At low peptide concentrations, recovery from down-regulation occurred rapidly, whereas with high concentrations, recovery occurred slowly or not at all.

Several biochemical mechanisms could explain this replenishment. If we speculate that receptor endocytosis accounts for down-regulation, the internalized receptor may be protected from intracellular degradation and subsequently reinserted into the plasma membrane. With this model, occupied receptors on the monocyte membrane would be internalized, deliver their bound ligand to some intracellular compartment, and then cycle back to the plasma membrane. Similar models have been proposed to explain receptor-mediated uptake of several ligands, including α_2-macroglobulin-protease uptake by a closely-related cell, the alveolar macrophage (30). Alternatively, a latent or cryptic pool of receptors may exist either intracellularly or on the membrane, which could be expressed after the inactivation of the original surface receptors. The latter two possibilities are supported by the observation that stimuli that cause lysosomal degranulation and requisite fusing of lysosomal membrane with plasma membrane can increase the amount of surface receptor (13, 31). Additionally, treatment of rabbit neutrophils with several aliphatic alcohols, particularly n-butanol, increased the number of surface receptors by up to threefold, suggesting that latent receptors can be expressed on the membrane (32). New protein synthesis did not appear to be involved in receptor recovery from down-regulation because inhibition of protein synthesis by cycloheximide did not alter replenishment of surface receptors (Fig. 13).

However, the capacity for peptide endocytosis and receptor replenishment must be limited because a plateau was reached during a time-course of peptide uptake (Fig. 2). If these processes were not limited, peptide uptake would be linear with time until the concentration of peptide in the medium were significantly decreased. Peptide depletion did not explain the plateau because sufficient native ^{125}I-labeled fNLPNTL remained in the medium to yield normal uptake values when applied to fresh cells. The data in Fig. 12 demonstrate that the cellular capacity to replenish surface receptors decreased when the length of the incubation used to induce down-regulation was increased. Comparable receptor down-regulation was achieved by a 15-, 30-, or 60-min incubation with 20 nM fNLPNTL. Whereas the cells down-regulated for 15 min displayed complete receptor recovery during a subsequent 60-min incubation, only 50% receptor recovery occurred under identical conditions with cells down-regulated for 60 min. This time-dependent loss of the capacity to replenish down-regulated surface receptors, coupled with the intracellular degradation and release of the internalized peptides observed previously (12), may explain the plateau and eventual

decrease in cell-associated ^{125}I-labeled fNLPNTL seen during the time-course of uptake experiments.

The biological relevance of the peptide-induced modulation of the human monocyte chemotactic receptor presented here or of similar receptor modulation demonstrated with human and rabbit neutrophils remains uncertain (12, 14, 28, 29). Attenuation of several cellular responses (termed deactivation) including chemotaxis (33), lysosomal enzyme release (34) and aggregation (34) is known to occur following exposure of phagocytic cells to the formyl peptides. The correlation between functional deactivation of monocyte chemotaxis and receptor down-regulation was inexact (Fig. 10). However, in light of our new understanding of receptor down-regulation and recovery, these experiments were ill-conceived. Pretreated cells will partially recover from down-regulation during the chemotaxis assay, but the rate and extent of this recovery will vary depending on both the time of incubation and the peptide concentration used to induce down-regulation. Additionally, during the chemotactic response, the cells are exposed to a continuously increasing concentration of peptide attractant and therefore increasing receptor down-regulation is presumably occurring during the assay. The measured chemotactic response may well be a function of these minute-to-minute changes in receptor down-regulation and recovery and chemotactic deactivation may in fact be the functional correlate of this complex receptor modulation. At present, no techniques are available to measure and integrate all of these variables affecting receptor number during the prolonged incubations required for the Boyden chamber chemotaxis assay. Other, more rapid, functional assays such as degranulation (13, 31, 34, 35) or orientation (36, 37) would be more suitable for these correlative experiments. Vitkauskas et al. (28) were able to show that deactivation of the degranulation response of rabbit neutrophils paralleled receptor down-regulation, although the percent decrease in the biological response consistently exceeded the percent decrease in receptor number. Thus, deactivation, which could play an important part in controlling phagocyte responses to environmental stimuli in vivo, may be mediated only in part by receptor down-regulation.

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