Consequences of Chemotactic Peptide Receptor Modulation for Leukocyte Orientation

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ABSTRACT To exhibit chemotaxis, the orientation of locomotion along a chemical gradient, cells sense differences in concentrations of a chemotactic factor by detecting some difference in the occupancy of their chemotactic receptors. Thus chemotaxis is sensitive to the number of receptors present and might be used to evaluate the consequences of receptor down-regulation.

The ability of rabbit peritoneal polymorphonuclear leukocytes (PMNs) to orient to a standard gradient at various concentrations of *N*-formylnorleucylleucylphenylalanine (FNLLP) was examined. The observed orientation was compared to that expected if the directional signal were proportional to a difference in the absolute number or the fractional number of receptors occupied. The receptor occupancy in varying gradients was calculated from the binding constant of FNLLP, 2×10^{-8} M (Zigmond and Sullivan, 1979, *J. Cell Biol.* 82:517–527), and the receptor number (*a*) present initially or (*b*) present after down-regulation (Sullivan and Zigmond, 1980, *J. Cell Biol.* 85:703–711). The observed concentration dependence of cell orientation is similar to the change in the number of receptors occupied, the receptor number being corrected for down-regulated cells. The net effect of receptor loss appears to be a decreased sensitivity to gradients at high concentrations of peptide.

Incubation of cells with a variety of different ligands results in the decrease of the number of receptors for that ligand on the cell surface. This process of ligand-induced receptor modulation has been called down-regulation (1). In most cases the consequences of this receptor loss for subsequent cell function are not clear.

When leukocytes are incubated at 37°C with chemotactic peptides, the number of their peptide receptors is reduced (2). This reduction is dose dependent, being greatest when cells are incubated in relatively high concentrations of peptide. When incubated in concentrations ~10 times the dissociation constant, K_d, of the receptor for the peptide, the receptor number is reduced to ~20% of control levels. The remaining receptors exhibit the same affinity for the peptide as do the original receptors (2). The receptor loss occurs rapidly after peptide addition, reaching a plateau level within 15 min. It has previously been demonstrated that the ability of human PMNs to orient in various gradients of the chemotactic peptide *N*-formylmethionylmethionylmethionine (fMetMetMet) is similar to that expected if the magnitude of the directional signal is a result of the difference in the number of receptors occupied over some distance in the gradient (3). From this one can predict that cells should orient optimally in a standard gradient (e.g., a 10-fold gradient across a 1-mm distance), when the concentration of peptide is at the K_d for its receptor. The orientation should decline symmetrically on a log plot of concentrations above and below the K_d.

We have characterized the ability of the chemotactic peptide *N*-formylnorleucylleucylphenylalanine (FNLLP) to bind and to induce orientation in rabbit PMNs. The optimal orientation occurs at concentrations below the K_d. The accuracy of the orientation decreases more rapidly at high concentrations of peptide than at low concentrations. This asymmetry was also seen with fMetMetMet.

Cells responding to a chemotactic peptide in in vitro assays (and presumably also in vivo) are exposed to the peptide for extended periods of time, e.g., several hours. Thus, the cells in these circumstances will have the number of their receptors reduced according to the concentration of peptide present. After correction for this receptor loss, the magnitude of the directional signal based on the change in the number of receptors occupied now fits closely with the orientation behavior observed experimentally. By extrapolating back to estimate the orientation if there were no receptor loss, we find that the net consequence of receptor loss is a deceased sensitivity to gradients at high concentrations of peptide.

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MATERIALS AND METHODS

Cells

Rabbit peritoneal exudate cells were collected 4 h after injection of 0.1 g of shellfish glycogen (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) in 250 ml of 0.9% saline. Contaminating erythrocytes were lysed by brief (60-s) treatment with hypotonic (0.18%) saline. The cells were washed twice with 0.9% saline and then resuspended in Hanks' Balanced Salt Solution (Grand Island Biological Co., Grand Island, N. Y.) buffered with 2.4 mg/ml of HEPES (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 3.3×10^6 cells/ml.

Orientation Assay

A visual chemotaxis chamber previously described (3) was used. 100 μl of 3×10^6 cells/ml was spread across the center of a 22 × 40 mm cover slip and incubated for 5 min to allow the cells to attach. The chamber was assembled by inverting a cover slip with cells attached across its center region onto the plexiglass slide and securing the cover glass in place with the clips. The two wells (each holding 100 μg) were then filled with different concentrations of chemotactic peptide in a buffer solution.

The cells in the center microscope field of the bridge were observed with a × 40 phase objective (field diameter, 0.4 mm) between 15 and 30 min after assembling the chamber. The direction of locomotion of the PMNs was judged morphologically; the front of a locomoting cell was identified by its pseudopod and the rear by its knoblike tail. Cells were scored as moving either toward the high concentration of a chemotactic factor or toward the low concentration. Only cells with pseudopods and tails and whose direction of movement could be evaluated were scored. Thus, immobile cells and cells moving perpendicular (≤10°) to the gradient were not counted. The unscored population was usually ~20% of the PMNs present. The bridge was scanned across the slide (in the direction normal to the gradient) until at least 50 cells were scored. The results were expressed as the number of cells moving toward the high concentration divided by the total number of scoreable cells (the sum of those scored as moving toward and away from the chemotactic factor) × 100.

Binding Assay

Petri dishes containing 10^7 cells were incubated in 0.5 ml of Hanks' with the appropriate concentration of tritiated (12.5 Ci/mM) FNLLP. After incubation, this medium was aspirated and the dish washed quickly (6 s) and vigorously in two baths of 4°C saline. The reversibility of binding was measured by varying the time of the wash in chilled saline. Cell-associated radioactivity was measured by adding 0.5 ml of 0.1 N NaOH to each dish. The cells were scraped into this fluid with a rubber policeman, and then each dish was washed with 0.5 ml of distilled water. This 1 ml of cell suspension was counted in 15 ml of scintillation fluid Formula 963 (New England Nuclear, Boston, Mass.) with an efficiency of ~40%. In preliminary experiments, Lowry assays were done to confirm that cells were not lost from any of the dishes during the treatment. In all experiments the dishes were monitored visually for cell loss.

Down-regulation

Cells were preincubated with unlabeled FNLLP for various lengths of time at 37° or 4°C. At the end of the incubation, the medium was aspirated and the cells were washed for 5 min at 4°C with chilled saline (5 × 2 ml washes). The rebinding capacity was measured by adding 0.5 ml of Hanks' with tritiated FNLLP for 15 min at 4°C. At the end of this incubation, the cells were washed quickly, as outlined above. Control cells were preincubated without peptide but were subjected to the same washing procedure.

RESULTS

The extent of receptor loss after 20 min at 37°C in various concentrations of FNLLP has now been determined in >40 different experiments. The percent receptors lost at various concentrations of peptide are: at 1×10^{-6} M, 85 ± 8% (mean ± SEM); at 3×10^{-7} M, 80 ± 2%; at 3×10^{-8} M, 56 ± 5%; at 1×10^{-8} M, 37 ± 5%; at 3×10^{-9} M, 10 ± 10%; and at 1×10^{-9} M, 4 ± 10%.

The affinity of the receptors before and after receptor loss is the same: $K_d = 2 \times 10^{-8}$ M (2). Thus the number of receptors occupied at various concentrations of FNLLP at 37°C, i.e., after down-regulation, and at 4°C, before down-regulation, are shown in Fig. 1. These are idealized curves for the noncooperative peptide binding with a K_d of 2×10^{-8} M in both cases (2, 4). The loss of receptors, which is particularly prominent at high concentrations, decreases the extent of binding to about one-fourth of that present originally.

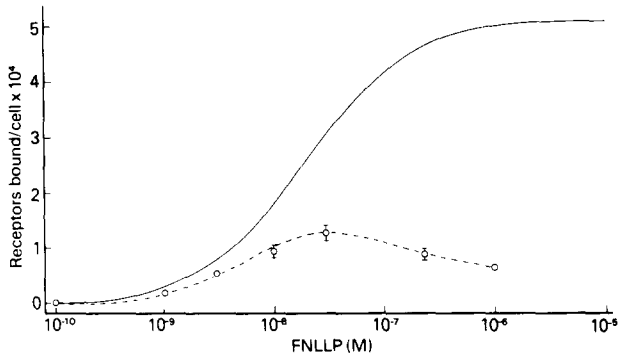

FIGURE 1 Plot of the receptors occupied as a function of concentration of FNLLP at 4°C, (solid line), or after receptor loss at 37°C, (dashed line). The 4°C curve (no down-regulation) was computed from information presented previously (2, 4). Receptor occupancy, $[RC]$, at any concentration $[C]$ is equal to $[C]/([C] + K_d) \times [R_o]$, where the dissociation constant K_d is 2×10^{-8} M and $[R_o]$, the total number of surface receptors, is 5×10^4/cell (2). The curve for receptor occupancy after down-regulation was computed in the same way using the reduced values of $[R_o]$ indicated in the text; the K_d remains at 2×10^{-8} M.

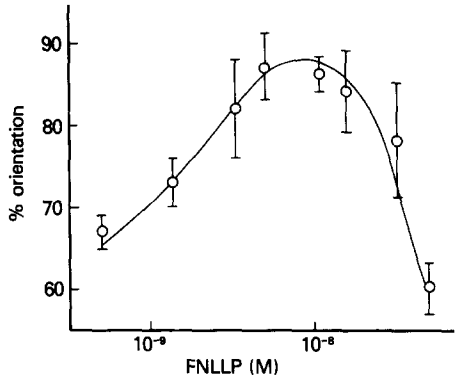

FIGURE 2 The percentage of PMNs oriented into the 180° sector toward the higher concentration of FNLLP is plotted vs. the mean peptide concentration over the 1-mm bridge. All tests were done with a 10-fold increase in concentration across the 1-mm bridge. The data is plotted as mean ± SEM.

As seen in Fig. 2, the ability of rabbit peritoneal PMNs to orient in various 10-fold gradients of FNLLP across a 1-mm bridge in a visual assay is optimal when the mean concentration is $\sim 1 \times 10^{-8}$ M. Although there is scatter to the data, several features appear consistently. The peak orientation occurs below the K_d of binding and the curve is not symmetrical but falls off more rapidly at high concentrations than at low concentrations. The asymmetry of the curve is also apparent when a different peptide fMetMetMet and human peripheral blood PMNs are used, as has been published previously (3).

In Figs. 3 and 4, this data is replotted as a heavy solid line (*b*). In addition, several other functions of the binding curve before (i.e., at 4°C, Fig. 3) and after (i.e., after 20 min with peptide at 37°C, Fig. 4) receptor loss are plotted. The curve of the number of receptors occupied as a function of concentration is shown as the dot-dash line (*a*). After receptor loss, 100% binding (Fig. 4) is equivalent to only ~20% of the original

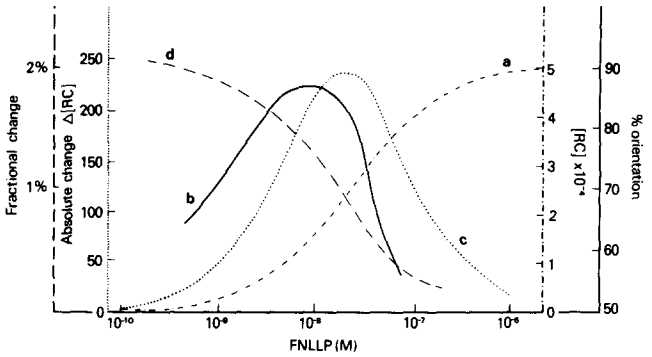

FIGURE 3 Comparison of orientation behavior in peptide gradient with various parameters of receptor occupancy existing before receptor loss. The number of receptors occupied at each concentration (dot-dashed line, *a*) is replotted from Fig. 1. The cell orientation observed at each concentration in a 10-fold gradient over 1 mm (solid line, *b*) is replotted from Fig. 2. The calculated change in the number of receptors occupied after a 10-μm displacement up the gradient from the center of a 10-fold linear gradient over 1 mm is plotted as a dotted line (*c*). This 10-μm displacement would result in ~2% increase in the mean concentration of peptide. Thus this is the difference in the number of receptors occupied between two concentrations, i.e., $[RC_2] - [RC_1]$. For example, the difference in number of receptors occupied between a cell at concentrations of 1.02×10^{-8} M and a cell at 1.00×10^{-8} M would be 16,887 − 16,667, or 220, receptors. The fractional change in the number of receptors occupied over the same change in concentration, i.e., $([RC_2] - [RC_1])/[RC_1]$, is shown in curve *d*. For a change in concentration between 1.0×10^{-8} M and 1.02×10^{-8} M FNLLP, the fractional change would be 220/16,667 or a 1.32% change. This can also be considered as the signal resulting from the change in the ratio of the receptors occupied at the two concentrations, because $([RC_2] - [RC_1])/RC_1 = (RC_2/RC_1) - (RC_1/RC_1) = (RC_2/RC_1) - 1$.

binding, as seen in Fig. 3. The dotted curve (*c*) in Fig. 3 indicates the relative magnitude of the directional information available to a cell if the cell receives this information through changes in the number of its receptors that are occupied. Thus, curve *c* plots the calculated difference in the number of receptors occupied as a result of a 2% change in peptide concentration, that is, the difference in receptor occupancy in two cells 10 μm apart in a linear gradient where the concentration increases 10-fold over 1 mm. This curve is calculated using 5×10^4 as the number of receptors and 2×10^{-8} M as the K_d of binding. This curve, which is the derivative of the binding curve, is symmetrical with the peak at the K_d. The dotted curve in Fig. 4 is a comparable plot but corrected for the decrease in receptor number resulting from down-regulation. The peak orientation occurs below the K_d of binding, and this curve is asymmetrical, falling off somewhat more rapidly at high concentrations. This curve fits fairly well with the experimental data.

The dashed curve in Fig. 3 is included to examine the possibility that a cell does not detect a gradient through differences in the number of occupied receptors but rather through fractional changes in the number of occupied receptors, i.e., through changes in the ratio of occupied receptors (5). As can be seen, this would result in an extremely high sensitivity at low concentrations that would continually decrease with increasing concentrations. This clearly differs from the observed behavior. Thus PMNs as well as bacteria appear to detect a gradient through a change in the number of receptors occupied (5).

Fig. 5 plots the calculated change in number of receptors occupied over a 10-μm distance at the center of a 10-fold gradient before and after down-regulation, as in Figs. 3 and 4 but this time to scale. The difference between the two curves illustrates the effect of down-regulation: there is a decrease in sensitivity at high concentrations. Down-regulation has little effect on the directional signal at low concentrations.

DISCUSSION

Leukocytes respond to chemotactic peptides as many cells respond to peptide hormones, by decreasing the number of receptors available for further binding (2). The significance of the decreased receptor number for the chemotactic peptides is unclear. It may serve to limit certain leukocyte responses, such as increased adhesiveness, to situations where there is a dra-

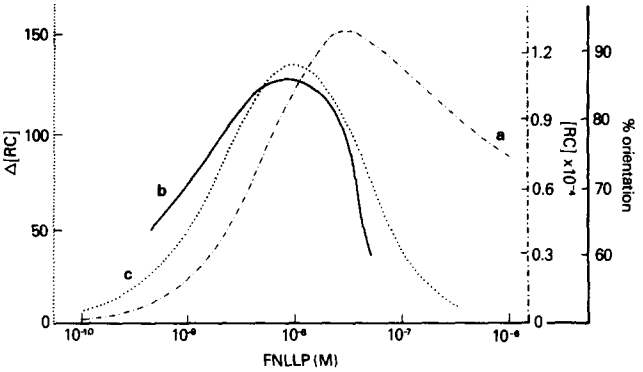

FIGURE 4 Comparison of orientation behavior in peptide gradient with various parameters of receptor occupancy that would exist after receptor loss. The number of receptors occupied at each concentration is the dot-dashed line (*a*; replotted from Fig. 1). The cell orientation observed at each concentration in a 10-fold gradient over 1 mm is the solid line (*b*; replotted from Fig. 2). The calculated change in the number of receptors occupied after a 10-μm displacement up the gradient from the center of a 10-fold linear gradient over 1 mm is shown in curve *c*. In contrast to Fig. 3 where the number of receptors occupied was calculated using $[R_o] = 5 \times 10^4$ receptors/cell, here $[R_o]$ is corrected for the receptor loss that occurs at each concentration as given in the text. For example, in 1×10^{-8} M FNLLP, where a 37% loss of receptors leaves $\sim 2.3 \times 10^4$ receptors/cell, the change in receptor number between a cell at 1.02×10^{-8} M and one at 1.090×10^{-8} M is 10,639 − 10,500, or 139, receptors.

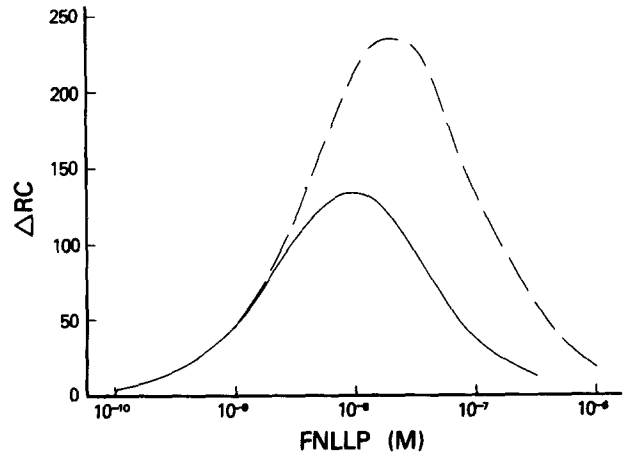

FIGURE 5 The calculated change in receptor occupancy over 10 μm in a 10-fold gradient with various mean concentrations before (dashed line) and after (solid line) down-regulation. The two curves are drawn to scale to illustrate that the net effect of down-regulation is a decrease in sensitivity at high concentrations.

matic increase in the concentration of the peptide. This could contribute to cells adhering to endothelial cells when suddenly exposed to chemotactic factors. The decreased receptor number could also limit the amount of an intracellular messenger to a small range around some sensitive threshold level. Alternatively, the decrease in receptor number may play no constructive role in the response but merely be a consequence of the relative rates of receptor-mediated peptide uptake and receptor reinsertion. From this study it seems unlikely that the receptor loss increases the chemotactic ability of cells. PMNs appear to sense the gradient by detecting a difference in the number of receptors occupied. The ability of cells to orient in a peptide gradient corresponds closely to that calculated for cells having undergone receptor loss. The orientation is markedly decreased at high concentrations of peptide (concentration greater than or equal to K_d) from that expected were there no receptor loss. The orientation data presented fit well with the directional signals calculated to be present.

The calculations assume a linear gradient across the bridge. We have previously demonstrated that an approximately linear gradient is established by ~15 min for a molecule with a molecular weight similar to that of the peptide (3). However, we have also noted that cells on the bridge appear to inactivate the peptide. It seems unlikely that inactivation of the peptide would lead to an orientation optimal at apparent concentrations lower than the K_d; rather, because the inactivation would cause the concentrations present to be less than those calculated, one might expect orientation to require higher concentrations. If digestion is playing a significant role, the down-regulation may not completely account for the observed behavior. Other peptide effects, such as alterations in the rate of locomotion by different concentrations of peptide, that greatly affect the outcome of many chemotaxis assays have little effect here because the orientation is independent of the rate of locomotion.

Berg and Purcell (6) have argued on theoretical grounds that little improvement in the ability to sense a concentration is achieved by increasing the receptor number above a/s specific receptors, where a is the radius of the cell and s is the radius of the binding patch on the membrane. For a PMN, a is ~10 μm and s can be estimated to be ~10Å. Thus a/s is $\sim 10^4$. It is interesting that even after maximal receptor loss there are still $\sim 10^4$ receptors (after 85% loss, there are $\sim 0.7 \times 10^4$).

That the orientation correlates with a change in number of receptors occupied over a given distance (e.g., 10 μm) does not define the specific details of how the cell senses a gradient. A cell could compare the receptor occupancy across its dimensions, for example, by comparing the density of receptor occupancy on its left and right. Alternatively, the cell could detect a change over time in the number of receptors occupied on small portions of its membrane. For example, if a pseudopod extends up a gradient the number of receptors occupied on its surface would increase. In either case, we can begin to get a feeling for the change in receptor occupancy that occurs when 90% of the cells viewed are moving into the 180° sector toward the well containing the higher peptide concentration. If we assume an equal distribution of the receptors on the cell surface, the number of receptors occupied on one-fourth of the cell surface can be compared with the number occupied by a similar portion of the cell surface 10 μm away. 90% orientation occurs in a 10-fold gradient between 2×10^{-9} M and 2×10^{-8} M over a 1-mm bridge; the concentration at the midpoint of the bridge is 1.1×10^{-8} M. The concentration 10 μm away is 1.12×10^{-8} M. At 1.1×10^{-8} M, 35.48% of the receptors are occupied, whereas at 1.12×10^{-8} M, 35.90% of the receptors are occupied. Here we assume there is no change in receptor number between these two concentrations and that ~60% of the original 5×10^4 receptors are present, i.e., 3×10^4 receptors/cell or 7.5×10^3 receptors per one-fourth of the cell surface. This results in a change of 32 receptors (2,693 − 2,661) being occupied. At 10^{-9} M, where a 10-fold gradient gives ~70% orientation, a change of only ~10 receptors would be expected. The changes during the extension of a 3-μm pseudopod would be even less. Clearly, the cell response depends on a relatively small change in receptor occupancy.

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