SHORT NOTE

Localization of Chemotactic Peptide Receptors on Rabbit Neutrophils

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The chemotaxis of blood leukocytes is initiated by the binding of a chemoattractant to specific receptors on the leukocyte cell surface [1, 2]. Although a great deal is known about the biochemical and morphological events accompanying chemotactic activation [3–9], there is very little morphological information about the chemoattractant receptors themselves. This latter information is needed so that we may understand the mechanism by which these inflammatory cells detect and respond to chemical gradients. One class of chemotactic factors extensively used to characterize the complex behavioral responses following leukocyte activation are the synthetic formylmethionyl peptides. These peptides, now known to be the analogs of the naturally occurring *N*-terminal peptides produced by bacteria [10], are released into culture medium and are believed to be responsible, at least in part, for the accumulation of leukocytes at the sites of bacterial infection [1, 2]. We have localized the receptors for the chemotactic hexapeptide *N*-formylnorleucyl-leucyl-phenylalanine-norleucyl-[^{125}I]tyrosyl-lysine [*N*-fNle-Leu-Phe-Nle-[^{125}I]Tyr-Lys] on whole rabbit peritoneal neutrophils (PMN) using light microscope autoradiography. By this method, the inherent formylpeptide receptor distribution on cells incubated at 4°C appears to be uniform over the surface of both rounded and structurally polarized PMN. Following a short 37°C incubation, cells retain a large proportion of labelled hexapeptide at or near the cell surface and, in addition, polarized PMN redistribute the hexapeptide anteriorly away from the cell uropod.

Rabbit peritoneal PMN obtained after glycogen infusion were centrifuged and resuspended in Hanks balanced salt solution containing 25 mM HEPES buffer and 2 mg/ml bovine serum albumin. Cell suspensions were placed onto acid-cleaned microscope slides, incubated for 5 min at 37°C, chilled to 4°C, and then exposed to 8.85 nM ^{125}I-labelled hexapeptide (approx. 1 000 Ci/mmole) at 4°C for 15 min. The slides were rinsed thoroughly in cold buffer and either fixed immediately (in cold cacodylate buffered 2% glutaraldehyde with 1% paraformaldehyde) or further incubated in buffer at 37°C for 2 or 10 min and then fixed. After fixation, slides and adherent cells were rinsed, dehydrated in graded ethanols, rehydrated in water, and coated with diluted Kodak NTB-2 emulsion. The autoradiographs were exposed for 3 days at 4°C, developed in Kodak D-19, air-dried, stained with toluidine blue, and mounted in Permount.

PMN fixed immediately following exposure to the chemoattractant at 4°C (fig. 1 *a*, *b*) display many silver grains (mean ± SE, 209±19). These grains appeared always in close proximity to the cell periphery as determined by focusing through

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Table 1. *Grain counts on [^{125}I]hexapeptide-labelled PMN*

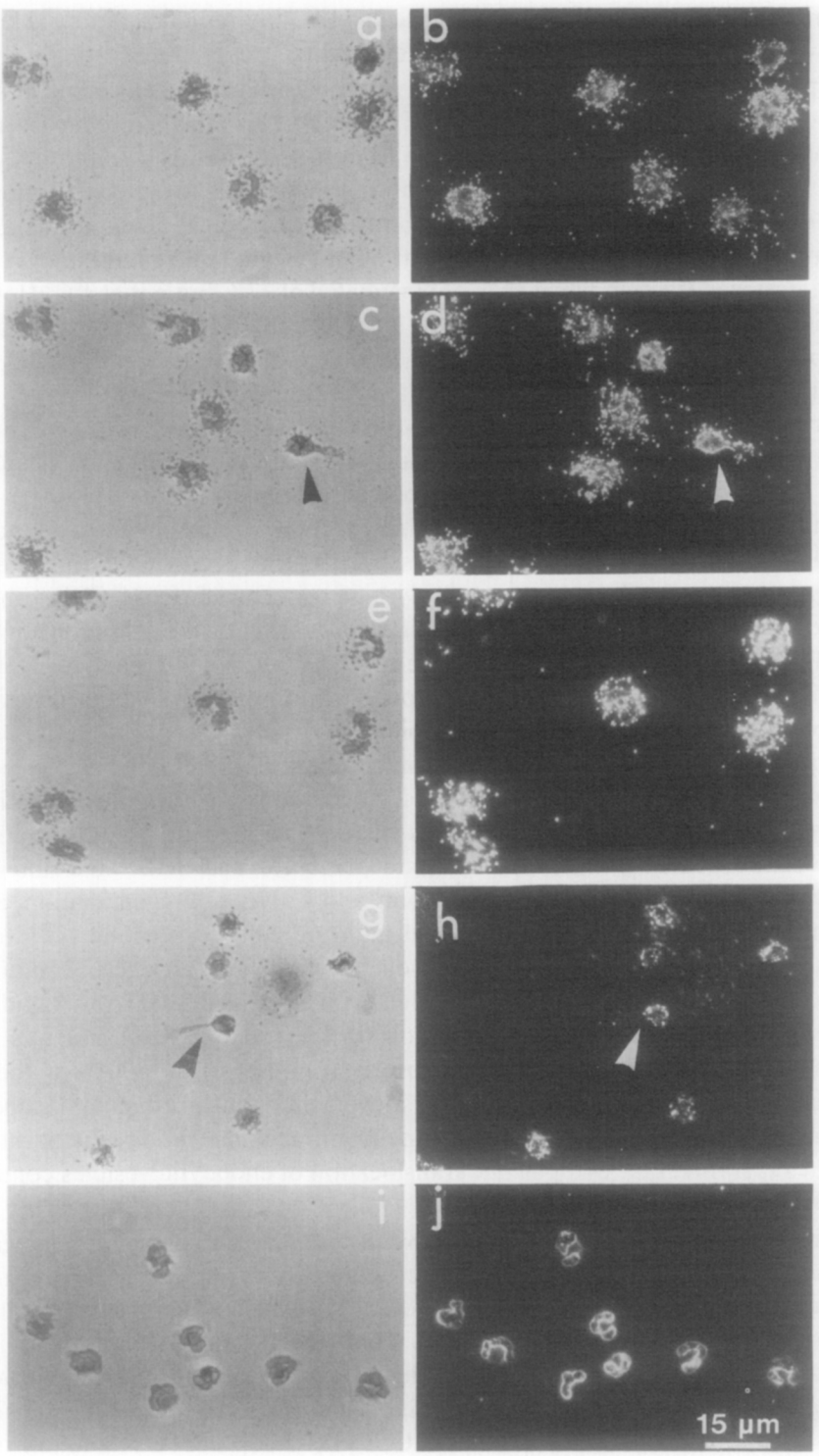
Treatment	N^a	Grains/cell^b	p-value^c
1. 15 min at 4°C/Rinse/Fix	9	209±19^b	–
2. 15 min at 4°C/Rinse 2 min at 37°C/Fix	9	174±10	NS
3. 15 min at 4°C/Rinse 10 min at 37°C/Fix	9	93±3	<0.001
4. 15 min at 4°C/Rinse 10 min at 4°C/Fix	9	102±5	<0.001

[a] Number of cells counted in each of two experiments.
[b] Results are expressed as means ± SE.
[c] Statistical significance was analysed by Student's *t*-test with the results from each treatment being compared with treatment 1.
NS, Not significant.

the cells. No grains were seen in association with the substrate-bound portion of the cells due to the absence of nuclear track emulsion in this region. Most PMN were rounded with grains distributed evenly over the entire upper and lateral portions of the cell. Some cells (<3%) were polarized in their morphology (probably motile) and these also exhibited an apparently uniform distribution of grains across their surface (fig. 1 *c, d*).

Cells labelled at 4°C, rinsed and then incubated at 37°C for 2 min (fig. 1 *e, f*) still displayed substantial numbers of silver grains. The grains were still distributed homogeneously and in close proximity to the cell periphery. The decrease in the average number of grains per cell after 37°C incubation (see table 1) may be the result of internalization of ligand receptor complexes and their transport away from the cell surface [11]. The internalized [^{125}I]hexapeptide may not be detectable by the method employed here. The resolution for low energy emissions by this method is approx. 0.1 μm [12] and the efficiency of grain production beyond 1 μm is very poor. Since high-energy emissions have a greater range but should

Fig. 1. Rabbit peritoneal PMN were exposed to *N*-fNle-Leu-Phe-Nle-[^{125}I]Tyr-Lys, fixed, and autoradiography performed as indicated in the text. (*a, c, e, g, i*) Toluidine blue-stained cells photographed using phase optics; (*b, d, f, h, j*) corresponding fields as seen by darkfield microscopy to accentuate developed silver grains. Rounded PMN fixed immediately following exposure to [^{125}I]hexapeptide (*a, b*) display many evenly distributed silver grains in close proximity to the cell surface. Spontaneously polarized PMN in these same preparations (*c, d, arrow*) also show apparently uniform distributions of grains across the cell surface. Cells labelled at 4°C, rinsed, and further incubated at 37°C for 2 min (*e, f*) exhibit somewhat fewer grains. After 10 min at 37°C, polarized cells display hexapeptide associated predominantly with the anterior portion of the cell (*g, h, arrow*). The coarse grains around the cell anterior (*h*) represent many very close-packed silver grains. Although most of the grains were found anteriorly, often a single grain was seen at the tip of the uropod. PMN incubated with ^{125}I-labelled hexapeptide together with a 20-fold excess of unlabelled hexapeptide show little evidence of bound radiolabel (*i, j*). Bar, 15 μm.


a
b
c
d
e
f
g
h
i
j
15 μm

contribute less than 20% of the grains seen [13], any radiolabel more than 1 μm below the cell surface should produce few grains.

A pronounced decrease in binding is also observed with cells labelled at 4°C, rinsed and then further incubated at 4°C (table 1). This most likely represents the dissociation of ^{125}I-labelled hexapeptide from the cell surface receptor (K_D=2.25 nM) since these conditions, i.e. (4°C), do not favor receptor internalization [11]. Jesaitis et al. [14] have shown, using photoaffinity-labelled chemoattractant and differential centrifugation, that chemoattractant is internalized and moves into a Golgi-enriched fraction when cells are incubated at 37°C but not at 4°C. However, the figures given for treatments 3 and 4 in table 1 (10 min at 37 or 4°C) cannot be compared to determine the amount of hexapeptide internalized at 37°C, since hexapeptide dissociation rates at these temperatures differ greatly.

Incubation at 37°C for 10 min resulted in increased numbers of polarized cells (<10% of total cells). This number of polarized cells is still quite low despite the 37°C incubation in the presence of bound chemoattractant. However, these experiments were designed to maximize receptor occupancy and intended to examine the temporal sequence of cell surface events occurring during chemokinetic activation. To this end, a relatively high concentration of [^{125}I]hexapeptide was employed initially at 4°C and cellular processing of the bound hexapeptide was followed for 10 min in the absence of additional soluble peptide. Optimum conditions for maximum chemotaxis occur when only a small percentage of receptors are occupied [15] and the ability to detect a chemotactic gradient as well as the ability to orient in a shallow gradient is greatest when the peptide concentration is in the region of the dissociation constant, K_D, of the peptide for the receptor [1, 16]. Our experiments were performed at a concentration of peptide above the K_D of the hexapeptide receptor and cells were allowed to interact with free, soluble hexapeptide only at 4°C. Upon 37°C incubation, excess unbound chemoattractant was removed allowing cells to respond only to previously bound hexapeptide. Under these conditions (i.e., absence of continuous soluble stimulus), hexapeptide associated with polarized PMN was rapidly excluded from the posterior uropod region (in less than 10 min) and was found extensively or completely shifted anteriorly (fig. 1*g*, *h*) in >90% of the cells examined (n=85). This shift may reflect movement of surface-bound hexapeptide within the plane of the plasma membrane or intracellular processing of internalized hexapeptide, i.e., cytoplasmic translocation of endocytic vesicles containing receptor–ligand complexes.

Using video intensification microscopy to visualize a fluorescent conjugate of this hexapeptide, Niedel et al. [17] reported that clustering and internalization of the peptide occurred in 2–4 min. However, ^{125}I-labelled hexapeptide bound to rabbit PMN did not appear to cluster. This may be due to differences in the sensitivities of the assays employed. Clustering of ligand on the cell surface or aggregation below the cell surface in pinosomes are both within the sensitivity range of fluorescence video intensification microscopy. The resolution of our

light microscope autoradiographs is probably too low to distinguish these events. Thus, clustering and internalization probably occurred in our experiments but were not resolved.

Control experiments in which a 20-fold excess of unlabelled hexapeptide was added along with the ^{125}I-labelled hexapeptide at 4 or 37°C showed virtually no silver grains (fig. 1*i*, *j*). Therefore, binding of [^{125}I]hexapeptide was specific, receptor-mediated, and inhibitable with non-radioactive hexapeptide.

The inherent distribution of Fc receptors and, to some extent, conA-binding sites on human PMN oriented in a chemotactic gradient is asymmetric [18–20]. These receptors are found predominantly on the anterior lamellipodium. This inherent asymmetry may confer some advantage in recognizing and binding particular ligands or reflect the composition of the surrounding membrane. However, as this study shows no such asymmetry exists for the hexapeptide receptor on spontaneously polarized PMN. Thus, the mechanism by which PMN sense *N*-fNle-Leu-Phe-Nle-Tyr-Lys gradient seemingly does not involve inherent asymmetries of the hexapeptide receptor itself on the cell surface.

In contrast, ligand–receptor complexes usually behave very differently than do unoccupied receptors. We [20, 21] and others [22] have shown that a variety of ligand–receptor complexes are redistributed (capped) to and internalized at the uropod region. On the contrary, formylpeptide receptor complexes do not seem to follow this pattern and appear to redistribute swiftly *away from* the uropod. This unusual response may help to perpetuate the existing polarization or stimulate locomotion by contributing to an asymmetric activation of the motile machinery of the cell.

The mechanism by which rounded, unstimulated PMN initially sense a chemotactic gradient to develop polarized morphology followed by chemotaxis is the subject of intense investigation. Several theories have emerged to explain the sensory mechanisms. One theory implies a 'temporal mechanism' by which an organism could sense the concentration of a chemotactic substance at one point, move a certain distance and sense it again, comparing this new level with the previous one [23]. This theory implies a simple memory system exists in the cell. A second theory suggests a 'spatial mechanism' by which an organism compares the concentration of a chemotactic substance at two or more locations on its body at one time. This theory implies that the organism is capable of sensing the chemical gradient across its own dimensions [23]. The latter may involve the cell's ability to sense a differential occupancy of chemotactic receptors such that more receptors are occupied on the leading front (or in the case of a rounded, yet unstimulated cell on the side of highest concentration) than on the trailing uropod or side of lowest concentration. However, the inherent distribution of receptors (whether occupied or unoccupied) may still be symmetrically distributed across the cell surface even in a polarized cell. Alternatively, but still consistent with the spatial mechanism is that the chemical gradient is detected by an asymmetric distribution of chemotactic receptors. This latter distribution has been described

using a hemocyanin–tripeptide conjugate [24]. However, such an extremely large, multivalent probe may be ill-suited for detecting or localizing surface receptors for chemotactic peptides. Further studies using chemoattractant gradients and EM autoradiography to study [^{125}I]hexapeptide binding and intracellular processing may aid in further resolving these alternatives.

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