Asymmetric Distribution of the Chemotactic Peptide Receptor on Polymorphonuclear Leukocytes

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ABSTRACT The distribution of chemotactic peptide receptors on polymorphonuclear leukocytes (PMNs) was visualized using tritiated chemotactic peptide, *N*-formylmethionylleucylphenylalanine, coupled to hemocyanin (HY-FMLP). This probe was biologically active and the number of HY-FMLP molecules bound to the cell in a saturable manner corresponded closely to the number of peptide receptors characterized for rabbit peritoneal polymorphonuclear leukocytes (Sullivan, S. J., and S. H. Zigmond, 1980, *J. Cell Biol.*, 85: 703–711).

Cells exhibiting locomotion have a polar morphology easily recognized in the scanning electron microscope. HY-FMLP bound to these cells was asymmetrically distributed with the highest density of HY-FMLP bound to the midregion of the cell. There were very few particles bound to the tail regions. The binding to the leading ruffles was variable but usually less than to the midregion. Addition of high concentrations of uncoupled FMLP eliminated HY-FMLP binding, confirming that the hemocyanin observed was a marker for the saturable chemotactic peptide receptor. The asymmetry in receptor distribution was seen on cells that had been stimulated by low concentrations of either FMLP or another chemotactic factor, leukotriene B_4. Thus, peptide binding to the receptor was not required for the development of the asymmetric distribution.

The low density of receptors in the tail region of the cell was consistent with the decreased responsiveness of the tail to chemotactic stimulation (Zigmond, S. H., H. I. Levitsky, and B. J. Kreel, 1981, *J. Cell Biol.*, 89:585–592). The receptor asymmetry may contribute to the polar behavior exhibited by polymorphonuclear leukocytes and would be expected to quantitatively modify the directional information available to a cell in a gradient of chemotactic peptide.

Membrane topography and the distribution of surface components are of general interest in a variety of cells, particularly cells with an obvious polarized morphology. An excellent example of this type of cell is the polymorphonuclear leukocyte (PMN).[1] PMNs are phagocytic cells that are capable of chemotaxis, that is, oriented locomotion in a gradient of a chemotactic factor. Cells stimulated to exhibit locomotion by either a gradient or homogenous concentration of a chemoattractant show a morphological polarity. This morphology is characterized by a region of ruffles or lamellipodia at the front of the cell, a midregion containing the nucleus, and a narrower tail region often associated with retraction fibers at the rear. Zigmond et al. (19) have demonstrated that this morphological polarity correlates with a behavorial polarity that biases responses of different parts of a cell to chemotactic stimulation. Consequently, we postulated that an asymmetric distribution of chemotactic receptors might be associated with the polarized morphology and could contribute to this behavioral polarity. Furthermore, previous studies show an asymmetric distribution of Fc and C3b receptors and concanavalin A-binding sites on locomoting PMNs (13–15).

To investigate the distribution of the receptors for chemotactic peptides, we prepared hemocyanin linked to *N*-formylmethionylleucylphenylalanine (HY-FMLP). This probe was then used as a marker for the receptor on cells examined in the scanning electron microscope (SEM). We demonstrate that HY-FMLP is a valid marker for the chemotactic peptide receptor and that the distribution of receptors is asymmetric. The density of receptors was variable in the front, greatest in midsection of the cell, and decreased dramatically in the tail region. In addition, our data suggest that

[1] *Abbreviations used in this paper:* HY-FMLP, *N*-formylmethionylleucylphenylalanine coupled to hemocyanin; PMN, polymorphonuclear leukocyte; SEM, scanning electron microscope.

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development of this asymmetric receptor distribution does not require binding of peptide to the receptor. Some of this data has been presented previously (12).

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, NY) in 250 ml of 0.9% saline. For SEM samples, cells in exudate fluid were allowed to settle on 15-mm glass coverslips. Cells used in all other studies were washed twice with 0.9% saline and resuspended in Hanks' balanced salt solution without bicarbonate (GIBCO Laboratories, Grand Island Biological Co., Grand Island, NY) buffered with 2.4 mg/ml of HEPES (Sigma Chemical Co., St. Louis, MO).

Coupling Procedure: Hemocyanin was purified from *Busycon canaliculatum* hemolymph (Marine Biological Laboratories, Woods Hole, MA). After a low-speed spin to remove debris, the hemocyanin was concentrated by high-speed centrifugation (90 min at 50,000 *g*). Concentrated hemocyanin was purified by passage through Sephadex G75 (Sigma Chemical Co.,) and Sepharose 2B (Sigma Chemical Co.) columns. The protein concentration was estimated by the procedure of Lowry et al. (6) and the hemocyanin was stored in 0.3 M NaCl.

Tritiated chemotactic peptide, *N*-formylmethionylleucylphenylalanine (New England Nuclear, Boston, MA, 46.4 Ci/mmol) was dried down under N_2 and redissolved in anhydrous dioxane. The specific activity was decreased to 66 mCi/mmol by the addition of unlabeled FMLP (Sigma Chemical Co.), which was also dissolved in dioxane.

The coupling procedure was similar to that used by Schultz et al (9). Activation of the carboxyl terminal of FMLP was carried out in dioxane using *N*-hydroxysuccinimide and dicyclohexylcarbodiimide (DCC) to promote the formation of reactive succinimide esters (17). This activation mixture was stirred gently at room temperature for 4 h. The activated peptide was added to hemocyanin in 200:1 molar ratio, and the coupling reaction was allowed to proceed for 16 h at 4°C. The hemocyanin was lightly fixed by the addition of 0.05% glutaraldehyde for 1 h at 4°C before the addition of peptide to minimize hemocyanin dissociation into subunits. The advantage of this coupling procedure was that addition of hemocyanin in an aqueous solution inactivates the water-insoluble dicyclohexylcarbodiimide thereby preventing coupling of hemocyanin to itself. The peptide can not cross-link with itself because it has no free amino group. Coupling efficiency using this method ranged from 5 to 10%.

At the end of the coupling reaction, 0.05 M glycine or 0.1 M $NaBH_4$ was added to quench the remaining glutaraldehyde and the solution was run on a Sephadex G75 column to separate uncoupled peptide and coupling agents from the hemocyanin. The void volume peak was concentrated and run on a Sepharose 2B column to remove aggregates composed of more than five monomers. The fractions were assayed for radioactivity and protein (OD_{280}) and the coupling efficiency was calculated. Typically, <10% of the hemocyanin was found in the aggregate peak after coupling. Recovery of protein and radioactivity was ≥90% after the Sephadex G75 and ≥70% after the Sepharose 2B.

Binding Studies: Cells were allowed to settle on 35 × 10-mm petri dishes (Falcon Plastics, Oxnard, CA) for 15 min at 23°C at 5×10^6 cells per dish. The cells were lightly fixed in 0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 5 min at 4°C. The medium was aspirated and remaining glutaraldehyde quenched by addition of 0.05 M glycine for 15 min at 4°C. Control experiments indicated that 85% of the saturable binding was retained after this fixation procedure. HY-FMLP was added to the cells in 0.3 M NaCl and binding was carried out for 30 min 4°C. At the end of this time, the cells were briefly rinsed (6–10 s) in chilled saline. Cell-associated radioactivity was measured as previously described (11). Nonsaturable binding was measured as the amount of HY-FMLP bound in the presence of 10^{-5} M unlabeled FMLP.

Millipore Assays: Millipore filter assays of cell migration were run as previously described (18). Millipore filters with a pore size of 3.0 μm and a diameter of 22 mm (Millipore Inc., Bedford, MA) were used in Sykes Moore chambers (Bellco Glass, Vineland, NJ). The solution to be tested was added to the lower part of the chamber and 6×10^6 cells in 0.9 ml are placed above the filter. 0.1% bovine serum albumin (Miles Laboratories, Elkhart, IN) was present in both solutions. The chambers were incubated for 60 min at 37°C, after which the filters are fixed and stained. The distance from the top of the filter to the furthest plane of focus containing at least two cells was measured in five randomly selected fields.

Scanning Electron Microscopy: Cells adherent to 15-mm glass coverslips were stimulated by low concentrations of either FMLP or leukotriene B_4 for 5–10 min at 37°C. These stimulated cells were fixed in 0.25% glutaraldehyde and binding of HY-FMLP was carried out as described in the previous section. After the 6-s wash, the cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 4°C followed by 5 min of postfixation in 1% osmium tetroxide in 0.1 M cacodylate. The samples were dehydrated through a methanol series and critical point-dried in CO_2 (DuPont Instruments, Sorvall Biomedical Div., Newtown). Dried samples were sputter-coated with 100Å of gold-palladium and examined in an AMR 1000 SEM or JEOL 100CX scanning transmission electronic microscope (AMR, Bedford, MA, or JEOL USA, Electron Optics Div., Peabody, MA).

Transmission Electron Microscopy: Hemocyanin was examined using negative staining after purification and after coupling to insure that it had not dissociated and to check for the presence of aggregates. A drop of the hemocyanin solution was placed on a 200-mesh grid for 30 s before addition of 1% uranyl acetate for 30 s. After staining, the excess fluid was drained off and the samples allowed to dry before examining them in a Philips 500 transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ).

RESULTS

Characterization of HY-FMLP

The hemocyanin-linked peptide was first characterized to document that it was a suitable probe for the chemotactic peptide receptor. Experiments were done to insure that (*a*) the hemocyanin had not aggregated during coupling, (*b*) the peptide was covalently linked to hemocyanin, (*c*) HY-FMLP bound saturably to the chemotactic peptide receptor, and (*d*) the HY-FMLP probe retained biological activity.

After coupling, HY-FMLP was run over a Sepharose 2B column to remove any large hemocyanin aggregates. As mentioned previously, <10% of the protein was found in the void volume aggregate peak. In addition, hemocyanin coupled to peptide was negatively stained with 1% uranyl acetate and examined using transmission electron microscopy. The multisubunit structure of hemocyanin monomers can be seen in Fig. 1. Note that the hemocyanin was of variable size which is probably due to the presence of small aggregates. (See Materials and Methods.)

Several controls were done to verify that all of the peptide associated with the hemocyanin was covalently linked. To test HY-FMLP for binding and biological activity, it was crucial to rule out the possibility of contamination of HY-FMLP by free peptide. HY-FMLP dialyzed extensively (1 wk at 4°C) against 0.3 M NaCl and 1% BSA or run several times over Sephadex G75 did not show any change in the coupling ratio of six FMLP per hemocyanin. In addition, none of the radioactivity could be extracted with methanol and >99% of the radioactivity was trichloroacetic acid-precipitable. The coupling ratio remained constant for the period of time the probe was used (2–3 mo).

Initial analysis of the ability of HY-FMLP to bind the chemotactic peptide receptor was frustrated by high levels of nonsaturable binding (>80% of total). In an attempt to decrease this high background, the ionic conditions and pH were varied and the effect of addition of BSA or gelatin was tested. Only high salt (0.3 NaCl) was effective; nonsaturable binding decreased to 30–40% of total binding. Consequently, binding of HY-FMLP was done in Hanks' balanced salt solution containing 0.3 M NaCl using cells which had been lightly fixed in 0.25% glutaraldehyde for 5 min at 4°C. Greater than 85% of control peptide binding was maintained using these fixation conditions.

A dose response curve of HY-FMLP binding is shown in Fig. 2. It was clear from these data that FMLP coupled to hemocyanin bound saturably to the chemotactic peptide receptor. The affinity of the receptor for FMLP coupled to hemocyanin, however, appeared to have decreased by two

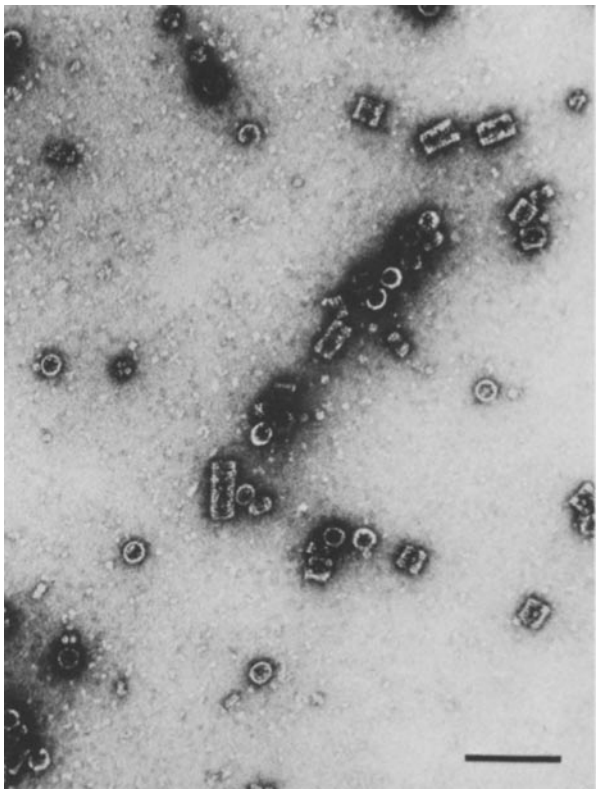

FIGURE 1 Transmission electron micrograph of whelk hemocyanin coupled to FMLP. The hemocyanin was negatively stained with 1% uranyl acetate. Bar, 0.25 μm. × 52,000.

orders of magnitude assuming that HY-FMLP is acting as a monovalent probe (see below). The dissociation constant, K_d, obtained from this dose-response curve was ~4 × 10^{-7} M (based on hemocyanin concentration). The K_d for uncoupled FMLP is 4 × 10^{-9} M (data not shown). This decrease in receptor affinity is likely to be due to steric considerations. The number of hemocyanin molecules saturably bound per cell roughly corresponded to the number of peptide receptors previously characterized for rabbit peritoneal PMNs (11). This suggested that although there are six molecules of FMLP covalently linked per hemocyanin, each ligand was bound to only one receptor. However, to rule out definitively the possibility of cooperative binding, the dose-response curve at concentrations well below that of the K_d would need to be investigated. The specific activity of the tritiated HY-FMLP probe was too low to be used for binding studies at these concentrations.

Once it was established that HY-FMLP binds to the peptide receptor, it was tested for biological activity. The ability of HY-FMLP to stimulate cell migration was tested using the Millipore filter assay (18). HY-FMLP and uncoupled FMLP were tested at concentrations equivalent to their respective K_d values and the results of this assay are shown in Table I. HY-FMLP showed levels of stimulation comparable to that of uncoupled FMLP. The equivalent concentrations of hemocyanin alone did not stimulate migration above control values. Thus, HY-FMLP retained biological activity that paralleled its binding activity.

SEM Analysis of Receptor Distribution

Exposure to chemoattractant, either in a homogeneous solution or in a concentration gradient, causes rounded cells to develop the polarized morphology characteristic of PMNs exhibiting locomotion. To determine receptor distribution on polarized cells, we incubated the cells for 5–10 min at 37°C in 10^{-9} M FMLP. This low peptide concentration is sufficient to stimulate the cells, yet the receptor loss due to down-regulation is <5% (11). To inhibit receptor redistribution which might be caused by HY-FMLP binding, we fixed the cells lightly in 0.25% glutaraldehyde for 5 min at 4°C, quenched with glycine as described previously, and then incubated for 30 min at 4°C in HY-FMLP. Higher concentrations of glutaraldehyde or longer times of fixation resulted in loss of saturable FMLP binding. At the end of this incubation, the cells were washed for 6 s. The hemocyanin-linked peptide was fixed in place with 2.5% glutaraldehyde and the cells were processed for SEM as described in Material and Methods. The fixation, dehydration and critical-point drying steps were all monitored for loss of radioactivity. In all examples shown losses were <10%.

HY-FMLP bound to a polarized cell is shown in Fig. 3. The inset in Fig. 3 shows a high-magnification view of he-

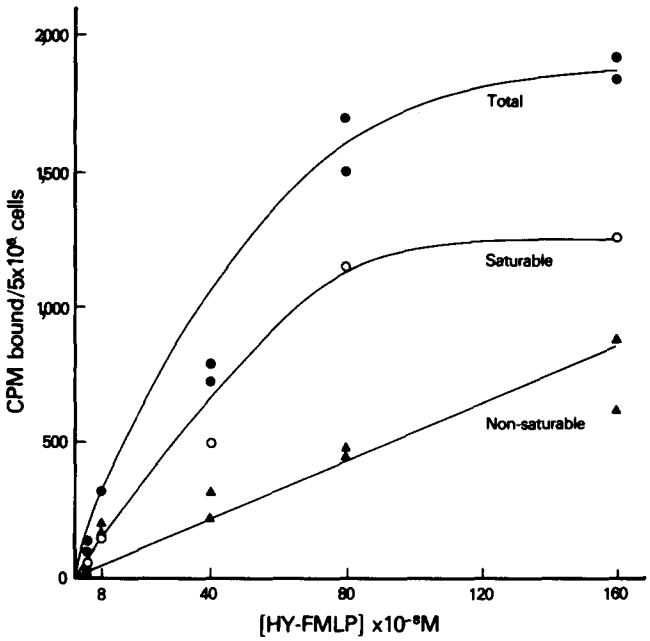

FIGURE 2 HY-FMLP binding dose-response curve. Rabbit PMNs adherent to petri dishes were incubated in various concentrations of HY-FMLP for 30 min at 4°C. The cells were briefly washed and assayed for cell-associated radioactivity. Nonsaturable binding was measured as the amount of HY-FMLP bound in the presence of 10^{-5} M unlabeled FMLP.

TABLE I

Biological Activity of HY-FMLP

	Distance moved into the filter	
	Exp. 1	Exp. 2
	μm	
BSA control	25	33
FMLP (4 × 10^{-9} M)*	83	104
HY-FMLP (4 × 10^{-7} M)*	77	87
Hemocyanin (4 × 10^{-7} M)	ND	38

ND, not done.

* These concentrations are equal to the K_d of the chemotactic peptide receptor for that ligand.

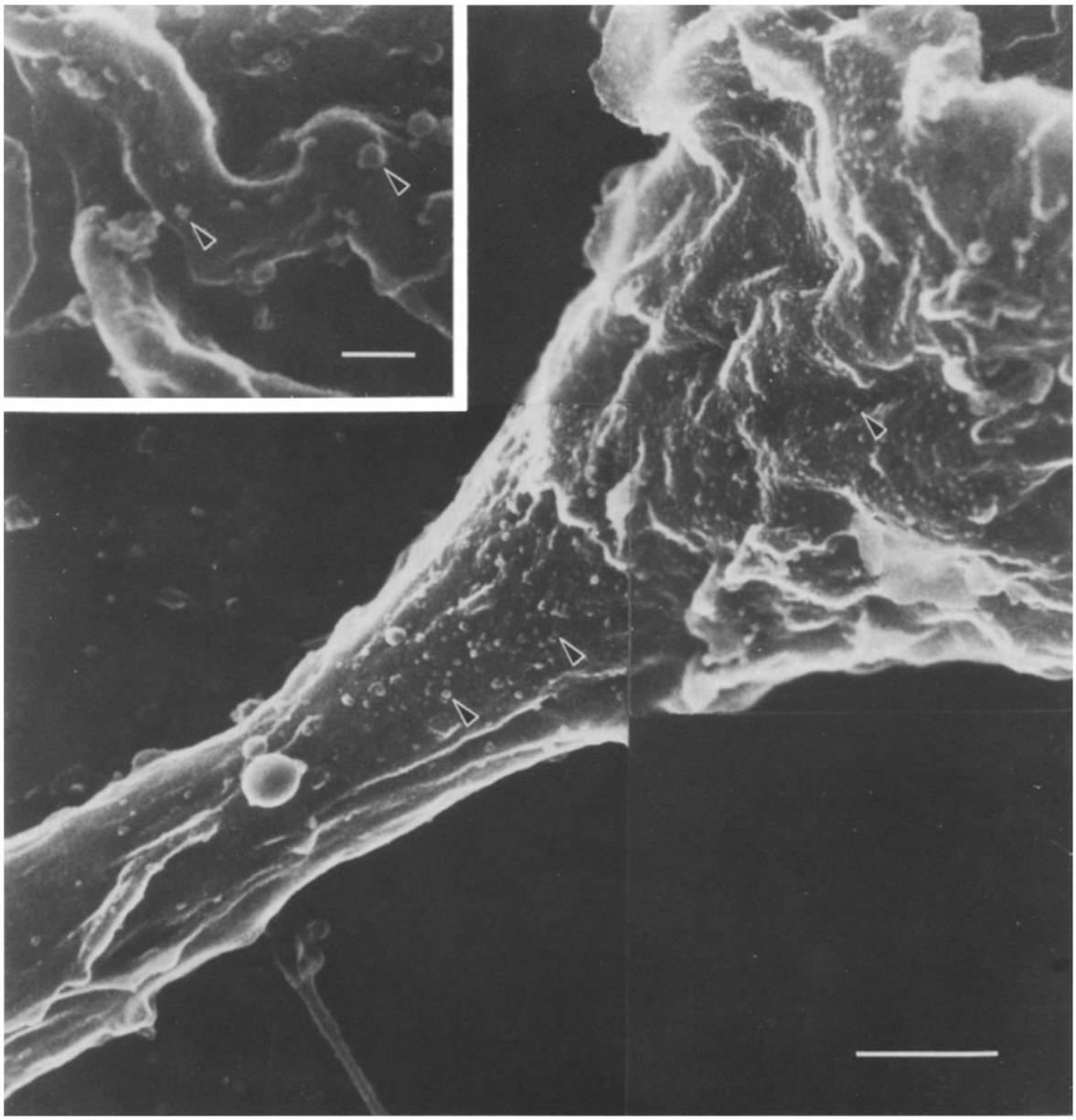

FIGURE 3 Scanning electron micrograph of locomoting PMN. Cells were stimulated with FMLP, lightly fixed, and then incubated with HY-FMLP and prepared for SEM as described. This composite illustrates the asymmetry in HY-FMLP binding. Substantially more hemocyanin can be seen bound to the front section of the cell than to the tail. Bar, 1.0 μm. *Inset*: A higher magnification view of HY-FMLP bound to the front ruffles. Hemocyanin molecules are indicated by arrows. Note that the hemocyanin is somewhat variable in size. Bar, 0.2 μm. × 21,000; *inset*, × 55,000

mocyanin on the cell. Arrows mark the hemocyanin which was somewhat variable in size when viewed in the SEM. In Fig. 3, two micrographs have been juxtaposed to show a view of a locomoting cell. There was a dramatic asymmetry in the receptor distribution with substantially less HY-FMLP bound to the tail region than to the more anterior parts of the cell. This very low density of HY-FMLP in the tail region was seen in all of the polarized cells which bound hemocyanin.

Morphometric analysis of the distribution of HY-FMLP is presented in Table II. The number of hemocyanin molecules per square micrometer was determined for the front ruffled area, the midsection, and the tail. Counts were done in areas that appeared to be perpendicular to the observer. It was apparent from these data that there were dramatically fewer HY-FMLP bound to the tail region and the binding of the hemocyanin to the midsection of the cell was always greatest. Binding to the ruffled region was the most variable and often low in the most anterior ruffles. A view of the front, ruffled

TABLE II

Morphometric Analysis of Distribution of HY-FMLP

Number of hemocyanin molecules per μm² of surface area*			
Cell	Front, ruffled region	Cell body	Tail
1	45.8 ± 6.2 (4)	53.6 ± 5.1 (5)	1.8 ± 1.2 (8)
2	60.7 ± 11.1 (6)	55.2 ± 7.3 (6)	0.6 ± 0.8 (7)
3	20.5 ± 11.6 (6)	41.2 ± 4.3 (5)	2.3 ± 1.9 (9)
4	24.8 ± 8.9 (9)	62.3 ± 11.8 (6)	2.8 ± 2.4 (9)

* Mean + standard deviation (*n* in parentheses).

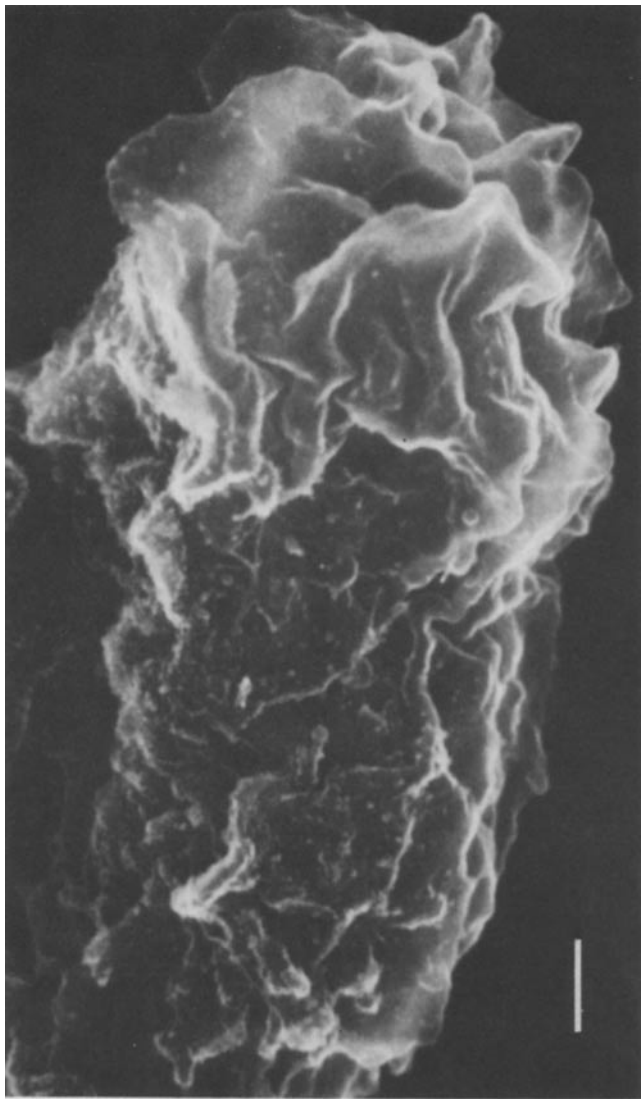

FIGURE 4 Binding of HY-FMLP to front and midsection of a stimulated cell. Note that HY-FMLP binding to the most anterior ruffles is low. Cells were processed as described in Fig. 3. Bar, 0.5 μm. × 26,000.

section and midsection of a stimulated cell is shown in Fig. 4. This micrograph illustrates the low HY-FMLP binding seen in the front section of the cell. In a given cell, there could be ruffles that were nearly free of hemocyanin as well as those which bound a density of hemocyanin similar to that of the midsection. The significance of this variability isn't clear. Although some variability may have been due to difficulties in counting in this highly irregular region, this can not explain the apparent lack of hemocyanin on some ruffles. It is possible that the newly formed pseudopods are free of receptors. Another possibility, which we can't eliminate, is that there may have been redistribution of HY-FMLP bound receptors even after the cells had been fixed in 0.25% glutaraldehyde and maintained at 4°C.

The data from HY-FMLP binding studies was used to predict how many hemocyanin molecules should have been seen on the cell surface. At saturation, we would expect ~100 molecules of hemocyanin per μm^2 assuming an even distribution of 5×10^4 receptors over an estimated 500–600 μm of cell surface (5). The number of hemocyanin molecules seen on the midsection of the cells was approximately one half of this value.

In contrast to cells exhibiting locomotion, round cells have random distribution of HY-FMLP on their surfaces. The asymmetry seen on polarized cells could have been developed in one of two ways. The cells were stimulated with FMLP, thus ligand binding to the receptor may have induced redistribution. Alternatively, asymmetric receptor distribution could occur with the development of the polarized morphology and be independent of the particular ligand bound. To distinguish between these two possibilities, cells were stimulated with a different chemotactic factor, leukotriene B_4, which does not compete with peptide for binding (4). Cells stimulated by 10^{-8} M leukotriene B_4 showed the same distribution of peptide receptors as cells stimulated by FMLP. This result indicates that development of locomoting morphology results in an asymmetric distribution of the chemotactic peptide receptor and that this receptor arrangement is not dependent on previous peptide binding.

There were some cells (both those with round and with polarized morphology) that showed little or no binding of hemocyanin to their surfaces. The percentage of cells that didn't bind HY-FMLP varied with different sample populations and could be as high as 20%. This suggests that rabbit PMNs from a peritoneal exudate are heterogenous with respect to their ability to bind chemotactic peptide. A similar heterogeneity has been reported for human blood PMNs (7).

Morphological analysis of HY-FMLP binding confirmed that HY-FMLP bound to the chemotactic peptide receptor in a saturable manner. Very little hemocyanin was seen on the surface of cells incubated with HY-FMLP in the presence of excess free FMLP. A cell incubated with HY-FMLP and 10^{-5} FMLP is shown in Fig. 5*A*. The nonsaturable binding of HY-FMLP to the glass substrate was clearly evident. However, a high magnification of the front of the cell documents that there was very little hemocyanin on the cell surface (Fig. 5*B*). Considering the previously mentioned heterogeneity in the ability of the cells to bind HY-FMLP, it was important to quantitatively substantiate this result. 90 cells from three different sample preparations were examined and none of them showed significant surface binding. These data corroborate the biochemical studies that indicated that HY-FMLP was a saturable marker for the chemotactic peptide receptor.

DISCUSSION

PMNs have a behavioral polarity that correlates with the morphological polarity seen in locomoting cells (19). Once the polarized locomoting morphology has developed, cells preferentially extend pseudopods from the front. Pseudopods rarely form from the tail regions: even when a gradient is reversed, cells usually reorient by extending pseudopods from the front and walking around in a circle (3, 19). In this study, we investigated whether the decreased responsiveness of the tail region reflects an asymmetry in the distribution of chemotactic receptors. By using the chemotactic peptide, FMLP, coupled to hemocyanin as a probe for the peptide receptor, we have demonstrated a dramatic difference in binding between the main body and tail of a polarized cell. Significantly less HY-FMLP bound to the tail regions than to the rest of the cell. A somewhat less dramatically decreased binding was seen in the front ruffles of a cell.

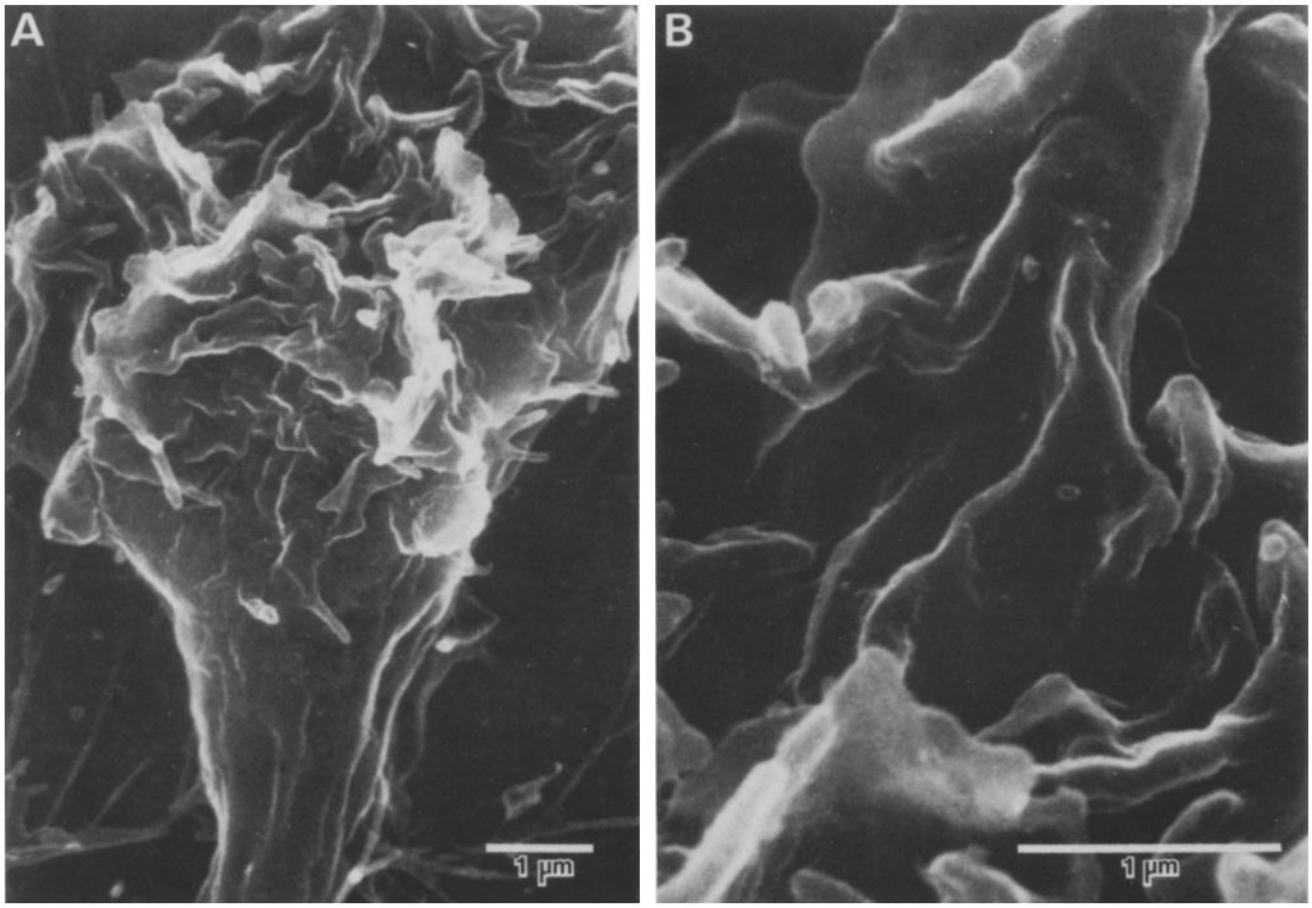

FIGURE 5 Competition for HY-FMLP binding by uncoupled FMLP. Cells, processed as in Fig. 3, were incubated with HY-FMLP in the presence of 10^{-5} M FMLP. (*A*) Whole cell, (*B*) front section of the same cell. Note that there is nonsaturable binding of hemocyanin to the substrate, but very little hemocyanin is seen on the cell. (*A*) × 15,000; (*B*) × 35,000.

The mechanism for the establishment and maintenance of this asymmetric receptor distribution is unknown. The low binding of HY-FMLP on the tail could arise from movement of receptors from the tail toward the front or from inactivation or internalization of receptors in the tail region. Davis et al. (2) have reported that pinocytic activity is restricted to the tail regions of polarized PMNs. They observed uptake of fluorescein-dextran by fluid-phase pinocytosis as well as receptor-mediated endocytosis of succinyl concanavalin A-receptor complexes almost exclusively in the tail region. In addition, we have indirect evidence suggesting that chemotactic peptide receptors are removed and rapidly recycled (20).

The variable binding on the ruffled regions of the cell is also interesting. It is possible that when ruffles first form they do not contain receptors for peptide and that receptors move out onto the ruffle. Alternatively, there could be some state of a ruffle, for example as it starts to withdraw, when receptors are removed. It is also possible that HY-FMLP binding induces some receptor redistribution which isn't completely blocked by our fixation protocol. Further experiments will be required to differentiate between the various possibilities.

The localization of peptide receptors to the front half of the cell was seen whether the cells were stimulated with low concentrations of FMLP or leukotriene B_4. Inasmuch as leukotriene B_4 does not compete for peptide binding (4), this result indicated that development of asymmetric receptor distribution did not require ligand binding. This is consistent with the observation that cells oriented in a peptide gradient still show a behavioral polarity in response to C5a which binds to a different receptor (1, 16). Preferential localization of Fc and C3b receptors and concanavalin A-binding sites at the leading edge of locomoting leukocytes has also been reported (13–15). Whether this receptor asymmetry is sufficient to account for the behavioral polarity is not clear. Several studies have demonstrated differences in distribution and organization of contractile proteins between the front and the tail of moving leukocytes (8, 10). These cytoskeletal elements may also contribute to the behavioral polarity and/or may be involved in stabilizing receptor distribution.

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