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28. A third action of SP, involving elevation of spike threshold, has only been observed with microiontophoresis of the peptide, whereas elevation of spike threshold by leucine-enkephalin has been observed with both iontophoresis and pressure application. Since we have also seen spike threshold elevation with iontophoresis of $H^+$ ions (D. L. Gruol, J. L. Barker, L. M. Huang, J. F. MacDonald, T. G. Smith, unpublished observations) further experiments are necessary to confirm this effect of SP.

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# Receptor-Mediated Internalization of Fluorescent Chemotactic Peptide by Human Neutrophils

Abstract. *Tetramethylrhodamine labeled* N-*formyl-Nle-Leu-Phe-Nle-Tyr-Lys is a potent chemoattractant for human neutrophils. Binding of this peptide to living neutrophils was observed by means of video intensification microscopy. At 37°C, diffuse membrane fluorescence was seen initially, followed by rapid aggregation and internalization of the fluorescent peptide. These processes are dependent on specific binding to the formyl peptide chemotactic receptor.*

Neutrophil chemotaxis is a complex behavioral response in which the cell exhibits directed migration in response to a chemical gradient. This implies that the cell is able to sense not only the concentration and chemical structure of the chemoattractant, but its directionality as well. Because of the vectorial nature of this signal, the molecular mechanisms of signal recognition or transduction may be more complex than that defined for other hormonally responsive cells, which must sense magnitude only.

Initial characterizations of the interaction of several chemotactic factors with neutrophils have demonstrated specific cell surface receptors for the factors, including formyl peptides, C5a (a complement-derived anaphylatoxin), and a urate crystal induced factor (*1*). In each case, the potency of a factor to induce chemotaxis correlated closely with its binding affinity for the specific receptor.

Neutrophils are believed to sense the directionality of a chemical gradient by a spatial, rather than a temporal, mechanism (*2*). The cell continuously determines the difference in concentration across its own dimensions. Because the chemotactic factors interact with cell surface receptors, it has been postulated that differential receptor occupancy across the cell membrane may account for recognition of directionality. Receptors nearest the source of the attractant, and therefore exposed to the highest relative concentration, would be occupied to a greater extent than those receptors on the same cell which are furthest from the source of attractant. In order to observe directly the binding and distribution of a chemotactic factor, we have prepared a fluorescent chemotactic peptide that retains biological activity. This peptide binds specifically to the plasma membrane of human neutrophils, rapidly aggregates, and is subsequently internalized.

Synthetic *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys [formyl peptide (*3*)], believed to be an analog of naturally occurring bacterial products that induce leukocyte chemotaxis, is a potent chemoattractant for human neutrophils, exhibiting an $EC_{50}$ (concentration producing a response 50 percent of maximal) for chemotaxis of 0.4 n*M* and $EC_{50}$ for binding of 1.3 n*M*. There are approximately 120,000 binding sites per neutrophil (*4*). Compared to the nonfluorescent formyl peptide, the potency of the tetramethylrhodamine conjugate of this peptide (TMR-peptide) (*5*) in inducing chemotaxis is only slightly decreased, the $EC_{50}$ being approximately 1.0 n*M*. The TMR-peptide also competes effectively for the formyl peptide receptor, exhibiting an $EC_{50}$ of 3.0 n*M* in a competitive binding assay.

Binding of the TMR-peptide to human neutrophils was observed by means of an RCA silicon intensifier target TV camera (TC1030H) attached to a Zeiss Photomicroscope III equipped with epifluorescence. Video output was recorded on a Panasonic recorder (NV-8030) and displayed on a Hitachi 8-inch TV monitor (VM-905AU), from which Polaroid photographs were taken (*6*).

Neutrophils that were exposed to TMR-peptide for 1 minute at 37°C displayed a diffuse and relatively homogeneous membrane fluorescence (Fig. 1, a and b). By 2 minutes, 20 to 50 aggregates of intensely fluorescent material could be seen superimposed upon the diffuse background fluorescence (Fig. 1, c and d). These aggregates were seen only in the focal plane of the plasma membrane, suggesting that the aggregates were confined to the membrane at this time point. By 3 minutes, these aggregates had increased in size and intensity on the membrane, and fluorescent endocytic vesicles, which displayed the saltatory motion characteristic of cytoplasmic organelles, were seen within the cell (Fig. 1, e and f). When cytoplasmic streaming was observed by phase-contrast, the fluorescent vesicles moved with the same velocity and direction as the cytoplasmic organelles. During the next 2 minutes, most of the fluorescent material remaining on the membrane was internalized and the numerous small endocytic vesicles coalesced into 5 to 10 larger vesicles (Fig. 1, g and h). The fluorescent vesicles correlated with easily definable, cytoplasmic lucent vesicles on the corresponding phase micrograph, suggesting that phase dense lysosomes had not, as yet, fused with the vesicles. In most cells, the coalescence of fluorescent vesicles continued, so that at 10 minutes, many cells displayed only one or two intensely fluorescent vesicles (Fig. 1, i and j). This pattern remained stable for 30 to 60 minutes. All of the neutrophils bound and internalized TMR-peptide to the same extent, providing direct evidence that the cells are homogeneous with regard to the presence of the formyl peptide receptor.

When the binding was carried out at 4°C for 120 minutes, the diffuse, homogeneous membrane fluorescent pattern was seen (similar to that in Fig. 1b). If the cells that had bound TMR-peptide at 4°C were then warmed on the microscope stage to 37°C, aggregation and internalization by individual cells could be followed. The time course and events were identical to those shown in Fig. 1.

To demonstrate that the rhodamine label being observed in Fig. 1 was attached to the original formyl peptide rather than to a hydrolytic product, we prepared a formyl peptide containing both $^{125}I$ and rhodamine in the same molecule


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([$^{125}$I]TMR-peptide) (*7*). After exposure to human neutrophils, this peptide rapidly aggregated and was internalized in a manner identical to the nonlabeled TMR-peptide. The cell-bound [$^{125}$I]TMR-peptide was then quantitatively extracted from cells that displayed aggregated (at 3 minutes and 37°C) or internalized (at 10 minutes and 37°C) fluorescence. The extracted peptide migrated with an $R_F$ value identical to that of the starting [$^{125}$I]TMR-peptide in three thin-layer chromatographic systems (*8*), and was able to bind in a saturable manner to the formyl peptide receptors of fresh neutrophils, demonstrating that the internalized fluorescent material was chemically and biologically identical to the starting peptide.

When cells were simultaneously exposed to TMR-peptide (2.5 n*M*) and nonfluorescent formyl peptide (100 n*M*), membrane-staining, aggregation, and internalization of fluorescent material were not observed (Fig. 1, k and l), demonstrating that the fluorescent events were dependent on specific binding to a saturable receptor rather than to fluid-phase pinocytosis. Aggregation and internalization were evident over a TMR-peptide concentration range of 0.1 n*M* to 10 n*M*, the same range that induced chemotaxis, suggesting that the processes are mediated by the same receptor. Human red blood cells, platelets, and lymphocytes, and 3T3 fibroblasts, which lack the formyl peptide receptor, did not bind or internalize TMR-peptide. The tetramethylrhodamine conjugate of the nonformylated analog, Nle-Leu-Phe-Nle-Tyr-Lys, did not induce chemotaxis or compete for the formyl peptide receptor in a competitive binding assay. This peptide did not fluorescently stain neutrophils at concentrations below 100 n*M*. However, above 100 n*M*, nonsaturable membrane fluorescence that did not aggregate and slow (>30 minutes) internalization was seen.

This study shows that the unoccupied formyl peptide receptors of the plasma membrane are diffusely and homogeneously distributed over the cell surface. Formyl peptide binding to cells at 4°C does not alter receptor distribution. However, warming these cells to 37°C or formyl peptide binding at 37°C causes a rapid aggregation of the fluorescent peptide (and possibly its receptor) into intensely fluorescent patches on or near the cell surface. These aggregates are quickly internalized by way of small endocytic vesicles, which then fuse into larger phase-lucent vesicles within the cell.

Whether these processes have any relevance to the chemotactic response is uncertain. Data from several investigators suggest that receptor aggregation may be an important mechanism of signal transduction. Binding of bivalent antibodies to the insulin receptor or immunoglobulin E (IgE) receptor will trigger many of the same biological responses caused by insulin or IgE binding. Monovalent antibodies are ineffective. In a similar manner, antibodies to insulin will enhance the biological re-

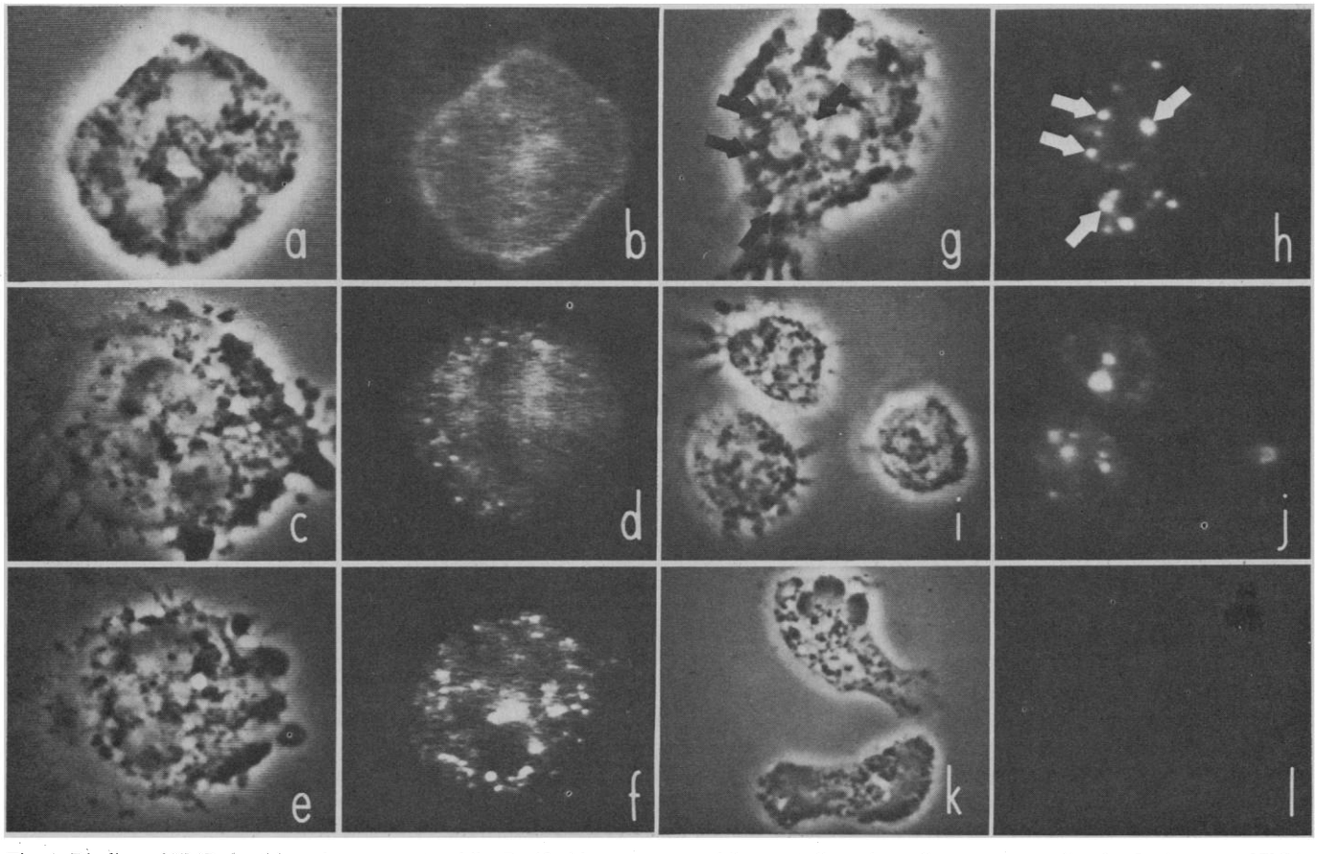


Fig. 1. Binding of TMR-peptide to human neutrophils. Purified human neutrophils were allowed to adhere to cover slips for 5 minutes at 37°C in Gey's balanced salt solution with 2 percent bovalbumin. The TMR-peptide was layered onto the cover slips to a final concentration of 2.5 n*M* and incubated at 37°C in humidified air containing 5 percent $CO_2$ for 1, 2, 3, 5, or 10 minutes. Binding was terminated by rinsing the cover slips in 4°C Gey's salt solution. Cells were observed through an oil immersion, Planapo 63/1.4 numerical aperture lens by phase contrast (a, c, e, g, i, and k) and fluorescent (b, d, f, h, j, and l) microscopy directly, or after fixation in 1 percent formaldehyde to ensure precise timing. Magnification on the TV monitor was ×10,000 for (a) to (h) and ×4,000 for (i) to (l). The incubation periods and treatments were as follows: (a and b) 1 minute, formaldehyde fixed; (c and d) 2 minutes, formaldehyde fixed; (e and f) 3 minutes, formaldehyde fixed; (g and h) 5 minutes, unfixed (arrows denote corresponding phase-lucent and fluorescent vesicles); (i and j) 10 minutes, unfixed; (k and l) 3 minutes, formaldehyde fixed [nonsaturable fluorescence was assessed by simultaneous exposure of the cells to TMR-peptide (2.5 n*M*) and nonfluorescent peptide (100 n*M*)].

sponse to suboptimal doses of insulin (*9*). Epidermal growth factor will induce receptor aggregation, whereas a competitive antagonist of epidermal growth factor will bind, but not cause, receptor aggregation (*10*). These studies suggest that aggregation of receptors or receptor-ligand complexes into groups of two or more may be essential for biological response.

Transduction of the chemotactic signal may also be dependent on aggregation of cell-bound peptide or peptide-receptor complexes into clusters of two or more. In this way, the intracellular signal would be proportional to the square or some higher power of the concentration of occupied receptors. This would serve to amplify small differences in receptor occupancy across a cell's dimensions when the cell is exposed to a concentration gradient of chemoattractant and may be important for the recognition of gradient directionality.

The role of internalization is also uncertain. Although only the TMR-peptide was seen to be internalized, studies with the $^{125}$I-labeled formyl peptide demonstrated the simultaneous loss of cell surface binding. This suggests that the peptide-receptor complex was internalized as a unit or that the surface receptors were inactivated by the initial binding event, perhaps because of masking by secretory or hydrolytic products or a conformational change leading to decreased affinity. The absence of cell surface binding persisted when the washed cells were incubated for 2 hours at 37°C. This process of internalization or inactivation may simply clear occupied receptors from the cell surface so that the cell can respond to changes in the orientation of the chemotactic gradient. Alternatively, the formyl peptides induce many other cell responses including superoxide production, lysosomal enzyme release, enhanced phagocytosis, and cell agglutination (*11*) which may be dependent upon internalization of the peptide or peptide-receptor complex. Receptor internalization or inactivation may explain the phenomenon of neutrophil deactivation, in which cells exposed to saturating concentrations of a chemotactic factor and subsequently washed extensively, are found to be unresponsive to the factor to which they have been exposed previously, but fully responsive to other, chemically dissimilar, chemotactic factors (*12*).

The TMR-peptide will facilitate several types of experiments. Binding to individual cells can be determined, thus the formyl peptide receptor can be assayed on cells that cannot be obtained in a pure form for radioligand studies. More important, occupied receptor distribution can be determined in the presence of a TMR-peptide gradient to test the differential receptor occupancy theory directly (*13*).

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8. Neutrophils ($10^6$ cells), which had been exposed to the [$^{125}$I]TMR-peptide for 3 minutes or 10 minutes at 37°C, were washed three times with 5 ml of phosphate-buffered saline (4°C) and extracted three times into 1.0 ml of a mixture of chloroform and methanol (1:2 by volume) with sonication and gentle warming. The purified [$^{125}$I]TMR-peptide and the extracted radioactive peptide were analyzed by thin-layer chromatography and autoradiography. They displayed identical $R_F$ values in the following mixtures: chloroform, methanol, and triethylamine (5:2:1), $R_F$ = 0.36; chloroform, methanol, and acetic acid (3:1:1), $R_F$ = 0.80; and *n*-butanol, acetic acid, and water (4:1:1), $R_F$ = 0.86.
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## Acetaminophen: Potentially Toxic Metabolite Formed by Human Fetal and Adult Liver Microsomes and Isolated Fetal Liver Cells

Abstract. *A reactive metabolite of acetaminophen is hepatotoxic in humans when the drug is ingested in large overdoses. The ability of the human fetal and adult liver to oxidize acetaminophen by trapping the potentially toxic metabolite as a glutathione conjugate has been measured. Oxidation by fetal liver was approximately ten times slower than by adult liver. However, there was a definite increase in acetaminophen oxidation with fetal age. Isolated human fetal liver cells conjugated acetaminophen with sulfate but not with glucuronic acid. The results indicate that the human fetal liver is able to detoxify acetaminophen by conjugation. However, it also catalyzes the formation of an active metabolite of acetaminophen through oxidation. Hence the fetus remains at risk should a large dose of the drug cross into the fetal circulation.*

The increasing number of drugs that are transformed into reactive intermediates in the body (*1*) raises the possibility of toxic effects on the exposed human fetus. The human fetus is at special risk from such drugs because, in contrast to fetuses from animals, it is able to oxidize drugs during the first part of gestation (*2*). Active metabolite formation has been proposed as the mechanism by which overdoses of acetaminophen, a widely used analgesic and antipyretic drug, produce centrilobular liver necrosis in humans (*3*). Studies in animals have shown that acetaminophen is oxidized by a microsomal mixed-function oxidase to a reactive arylating intermediate normally detoxified by conjugation with glutathione (GSH). If liver stores of GSH become severely depleted the intermediate binds with cell macromolecules and presumably results in cell death (*4*). A high-pressure liquid chromatographic (HPLC) method has been


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