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Cell Biology

Polymorphonuclear leukocyte histamine receptors: Occurrence in cell surface clusters and their redistribution during locomotion

(fluorescence intensification microscopy/adherence/chemotaxis)

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ABSTRACT **A univalent and bioactive fluorescent derivative of histamine bound to the surface of human polymorphonuclear leukocytes; free histamine was found to compete with this derivative for binding sites. Histamine H2-receptor specificity was indicated by binding inhibition experiments using cimetidine (H2-specific) but not diphenhydramine (H1-specific). Video-intensification fluorescence microscopy was used to determine the distribution of histamine receptors in living polymorphonuclear leukocytes. Receptors appeared as randomly distributed clusters upon stationary cells. During random locomotion, receptors were restricted to the ends of pseudopods, whereas chemotaxis led to receptor localization at lamellipodia and uropods. Ligand–receptor complexes were restricted to the cell surface, as shown by quenching exterior fluorescence with crystal violet. Therefore, pinocytic uptake cannot account for the observed receptor localization or clustering. As a further control, the lipid analog 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine remained uniformly distributed during all conditions. Histamine-mediated inhibition of adherence may be related to formation of ligand–receptor membrane domains at adherence sites.**

Histamine is a well-known vasoactive amine that is released during the inflammatory component of acute allergic responses (1, 2). Several laboratories have variously reported that histamine modulates polymorphonuclear leukocyte (PMN) chemokinesis, chemotaxis, degranulation, oxidative metabolism, and adherence (3–10). The broad spectrum of physiological reactions mediated by histamine are triggered by cell surface receptors (11, 12). However, the cell surface topography of histamine receptors and their modulation during distinct cellular activities are not known. Further, a suitable ligand to obtain this information has been lacking (13, 14, 29, 30). We have therefore synthesized a univalent and bioactive fluorescent derivative of histamine that binds with high specificity to the surface of living human PMNs. In addition to providing fresh information regarding cell surface properties of histamine receptors with a new fluorescence tool, our studies suggest a possible structure–function correlation, since the fluorescein-histamine conjugate (Flu-Him), which inhibits adherence, accumulates at sites generally associated with adherence activity.

MATERIALS AND METHODS

Preparation of Flu-Him. The side-chain nitrogen of histamine was converted to a secondary amine by reaction with fluorescein isothiocyanate (FluNCS) in ethanolic NaOH. Histamine and FluNCS were dissolved in 1:1 (vol/vol) ethanol/0.1 M NaOH and allowed to react in a light-tight test tube for 48 hr at 4°C. Histamine and FluNCS alone were treated in the same fashion as controls. Samples were acidified and then analyzed by thin-layer chromatography using 3:1:1 chloroform/methanol/ethanol as developing solvent. Analysis of the chromatograms revealed R_f values of 0.45, 0.55, and 0.81 for histamine, Flu-Him, and FluNCS, respectively.

Preparation of Cells. PMNs were obtained from clot preparations as described (15, 16). This preparatory method was chosen because it minimizes perturbation of cell function caused by handling and purification procedures. Drops of fresh blood were placed on glass coverslips in a humidified atmosphere at 37°C for 30 min, followed by gentle rinsing with 0.9% NaCl to remove the clot. These preparations contained 92–95% neutrophils, ≤3% eosinophils, and 4–8% monocytes. Adherent PMNs were employed for further experimentation.

Cell Adhesion. Cell adhesion was measured by the method of Keller *et al.* (17). Neutrophils were isolated from human peripheral blood according to the method of Ferrante and Thong (18). The Ficoll-Hypaque solution was obtained from Packard Instrument (Downers Grove, IL). Neutrophils at 10^6 per ml in Hank's balanced salts solution (HBSS; GIBCO) were incubated in tissue culture chamber/slides for 15 or 30 min at 37°C. The slides were thoroughly washed, fixed with ethanol, and then stained with Giemsa stain. Cell counts per unit area were made in the central region of the slide. Cells were untreated or treated with histamine or Flu-Him. Adherence is given as % inhibition in comparison to matched controls.

Chemotaxis. Chemotaxis chambers were constructed as described by Zigmond (16). *N*-formylmethionylleucylphenylalanine, at 1 μM in HBSS plus 2% heat-inactivated fetal bovine serum, was used as attractant. In some cases, coverslips were removed from the chamber, and the cells were fixed with 2% paraformaldehyde in 0.9% NaCl for 3–5 min. Samples were then labeled as described below. For studies with living cells, receptors were labeled as described below followed by insertion into the Zigmond chamber. In all cases, the chamber was kept at a nominal temperature of 37°C by use of an Incu-stage incubator (Lab-Line Instrument, Melrose Park, IL).

Cell Labeling. Cells were labeled with Flu-Him or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine ($diC_{18}Icc$) on coverslips. Flu-Him at 25 μM in 4:6 ethanol/H_2O was diluted in Dulbecco's phosphate-buffered saline (PBS) to various final concentrations prior to application to coverslips. In typical experiments, 10 μM Flu-Him was used to label cells for 5 min at 4°C. The reagent $diC_{18}Icc$ perchlorate was obtained from Molecular Probes (Junction City, OR). For $diC_{18}Icc$ labeling, 10 μl of a 300 μM solution in ethanol was diluted into 1 ml of PBS. Labeling was performed for 5

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Abbreviations: Flu-Him, fluorescein-conjugated histamine; $diC_{18}Icc$, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; PMN, polymorphonuclear leukocyte.

min at room temperature. Samples were washed three times with PBS.

Fluorescence Microscopy. Cells were examined in a Zeiss fluorescence microscope equipped with special excitation and detection instrumentation. The device employs a combination of fluorescence recovery after photobleaching and video-intensified microscopy (19, 20). An argon-ion laser (model 164-09; Spectra-Physics, Mountain View, CA) operating at 488 nm or 514 nm was used for excitation of Flu-Him or $diC_{18}Icc$, respectively. The intensity was adjusted by plasma tube current density, neutral density filters, and a beam-splitter assembly (19). The beam was spatially filtered, expanded (Ealing, South Natick, MA), and reflected into the epifluorescence port. A light level was chosen that did not photobleach the sample. A Leitz ×50 [numerical aperture (N.A.) = 1.0] or ×100 (N.A. = 1.2) water-immersion objective was employed. In these experiments, the image was reflected onto an RCA silicon-intensifier tube held in a Dage-MTI model 65 camera. Video signals were recorded on a Panasonic NV-8050 high-resolution video recorder and displayed on an Audiotronics monitor. The photographs reported were taken from the screen, using a Polaroid camera.

RESULTS

The bioactivity of Flu-Him was tested by using a previously described adherence assay (17). These experiments showed 55 ± 4% and 55 ± 7% adherence of PMNs to glass coverslips in comparison to controls in a 15-min assay at 10 μM histamine and Flu-Him, respectively.

After exposure of PMNs to Flu-Him (10 μM) at 4°C for 15 min, fluorescence was distributed in clusters (Fig. 1 *a* and *b*). Maximal binding of Flu-Him to PMNs was found at 10 μM. This is in good agreement with the results of Osband *et al.* (11), obtained using radiolabeled histamine. The clusters were predominantly associated with the cell surface, as judged by moving the focal plane through the cell (for additional controls, see below). The clusters cannot be due to extracellular crosslinkage, since Flu-Him is univalent. Binding was specific, since inclusion of a 100-fold excess of histamine abrogated binding (Fig. 1 *c* and *d*); in addition, this controls for bulk-phase pinocytic uptake (but not receptor-mediated uptake; see below) of Flu-Him and nonspecific membrane permeation. Both of these phenomena were undetectable. We have also tested the effects of cimetidine, an antagonist specific for H2 histamine receptors, on Flu-Him binding to PMNs. When PMNs were treated with 10 μM Flu-Him in the presence of 1 mM cimetidine, no labeling was observed (Fig. 1 *e* and *f*). The H1 antagonist diphenhydramine (at 1 mM) had no observable effect on Flu-Him binding (data not shown). These results are in agreement with previous reports (2) indicating the H2 specificity of the PMN histamine receptor.

Cell locomotion and polarization result in profound alterations in the distribution of histamine receptors at the PMN surface. Fig. 1 *g* and *h* show a PMN undergoing chemokinesis in the presence of Flu-Him. Ligand–receptor complexes were found at the ends of pseudopods and microvilli. Fluorescence was not found in association with the cell body. Under these conditions, some PMNs spontaneously polarize (Fig. 1 *i* and *j*). In this case, receptors were associated with the leading edge and uropod. Polarization and chemotaxis were induced by gradients of f-Met-Leu-Phe (16). Fluorescence was associated with the lamellipodium and/or uropod (Fig. 1 *k* and *l*) after fixation with 2% paraformaldehyde for 3–5 min. In the absence of fixation, receptors were rapidly redistributed to the uropod (Fig. 1 *m* and *n*). In the presence of EDTA, PMN locomotion but not polarization is inhibited. Again, fluorescence was found at the lamellipodium and/or uropod (Fig. 1 *o* and *p*). Small membranous vesicles arising from retraction fibers at the uropod were also labeled (Fig. 1 *p*).

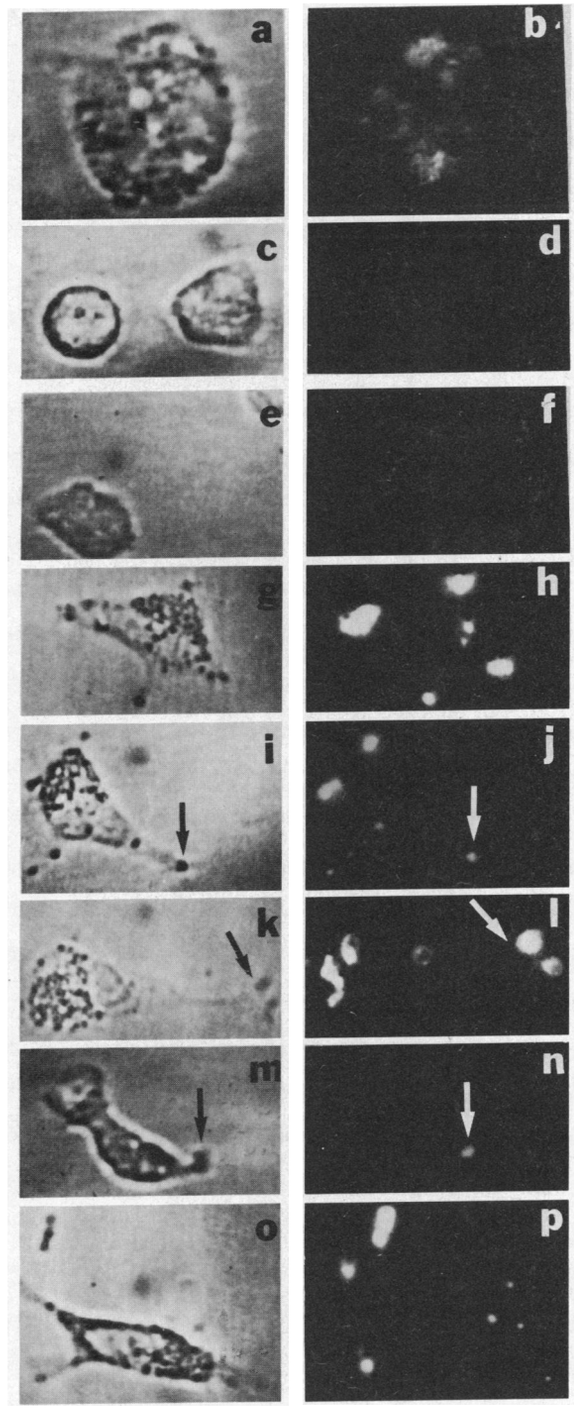

FIG. 1. Binding of Flu-Him to human PMNs. Adherent PMNs in Hank's balanced salts solution were observed. Samples were treated as described in the text with Flu-Him at 10 μM, followed by three washes with buffer. Bright-field (*Left*) and fluorescence (*Right*) images were recorded. These photomicrographs show a stationary PMN (*a* and *b*), PMNs with excess histamine (*c* and *d*), a PMN with cimetidine (*e* and *f*), a PMN during chemokinesis (*g* and *h*), a PMN spontaneously polarized (*i* and *j*), a PMN polarized during chemotaxis and fixed with 2% paraformaldehyde (*k* and *l*), a PMN polarized during chemotaxis without fixation (*m* and *n*), and a PMN polarized with f-Met-Leu-Phe in the presence of EDTA to inhibit locomotion (*o* and *p*). The areas of the uropods are indicated (arrows). (*a* and *b*, ×1900; *c–p*, ×950.)

In Fig. 2 we provide additional controls for the cell surface

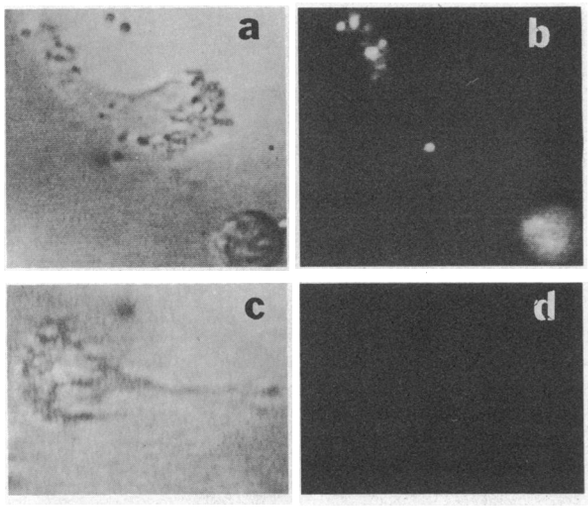

FIG. 2. Controls for pinocytic uptake of Flu-Him are shown. Samples were prepared as in Fig. 1. Bright-field (*a* and *c*) and fluorescence (*b* and *d*) images were recorded. (*a* and *b*) Control trial in the absence of crystal violet. (*c* and *d*) Cells in the presence of crystal violet. The quenching of fluorescence indicates that the Flu-Him is accessible to the extracellular medium. (×950.)

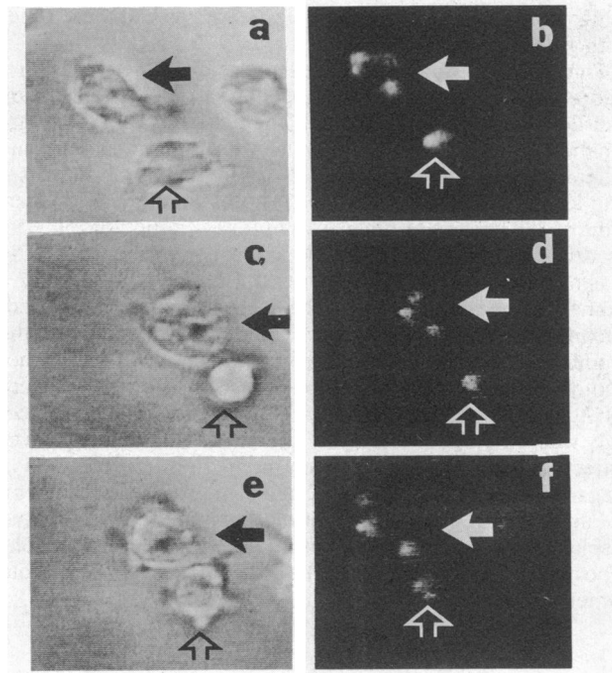

FIG. 3. Time-dependent changes in the distribution of Flu-Him. Cells were labeled with Flu-Him and exposed to f-Met-Leu-Phe as described in the text. Bright-field (*Left*) and fluorescence (*Right*) photomicrographs are shown. (*a* and *b*) Cells were observed roughly 1 min after labeling. The upper cell is indicated by solid arrows and the lower cell by open arrows. (*c* and *d*) Cells 210 sec after initial observation. (*e* and *f*) Cells at 290 sec. Cell morphology and the distribution of cell-associated fluorescence changes as a function of time. (×950.)

localization of the probe Flu-Him. The punctate appearance of the probe might be accounted for by receptor-mediated micropinocytosis of the ligand. Optical microscopy may not be sufficient to distinguish between these possibilities. However, the crystal violet quenching technique of Hed (21) provides a convenient tool to distinguish bound and internalized substances (21, 22). Fig. 2 shows bright-field and fluorescence photomicrographs of cells labeled with 10 μM Flu-Him in the absence (*a* and *b*) or presence (*c* and *d*) of crystal violet (4 mg/ml). In the latter case, no fluorescence can be observed. This shows that the probe Flu-Him was quenched by the reagent crystal violet, indicating an extracellular disposition (21). In similar experiments in this laboratory using fluorescent antigen–antibody complexes, substantial internalization has been found (for examples, see ref. 22).

In Fig. 3 we show time-dependent changes in receptor arrangement. Cells on coverslips were labeled on ice, washed with cold buffer, inverted onto a glass slide with an f-Met-Leu-Phe gradient, and then placed on a microscope stage at 37°C. Cells were microscopically followed with intermittent exposure to minimal levels of laser light. The photomicrographs of Fig. 3 show changes in the distribution of Flu-Him–receptor complexes with time. In this series a cell becomes polarized and begins to move (elapsed time is 4 min, 50 sec). The distributions of fluorescence seen in *b* and *f* are distinctly different. The relative changes in arrangement of fluorescence clusters cannot be accounted for by cells spinning in the dimension perpendicular to the focal plane; for this reason, a cell showing three clusters is advantageous. The upper cell moved roughly 10 μm. Longer time points are difficult to obtain due to ligand dissociation.

In Fig. 4 we provide control studies for membrane topographic reorganization. PMNs were labeled with $diC_{18}Icc$ as previously described (19). This probe (*i*) is not expected to be associated with the cytoskeleton, (*ii*) is a membrane marker, and (*iii*) provides a control for redundant or convoluted surface membrane, which could be misinterpreted as enhanced labeling when optical microscopy is used. Fig. 4*A* shows a stationary cell; the cell body and microvilli are uniformly labeled. Fig. 4 *B* and *C* show PMNs during chemokinesis and chemotaxis labeled with $diC_{18}Icc$ in the

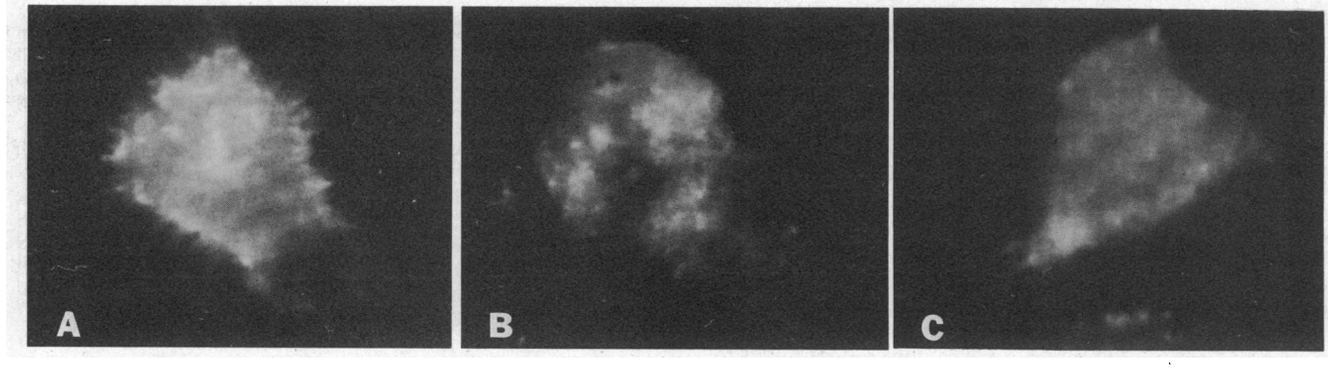

FIG. 4. Binding of $diC_{18}Icc$ to human PMNs. $diC_{18}Icc$ was incorporated into the cell membrane as described (19). (*A*) Cells were incubated with $diC_{18}Icc$. (*B*) Cells were treated with $diC_{18}Icc$ after exposure to 10 μM histamine. (*C*) Cells, polarized and migrating due to exposure to a gradient of f-Met-Leu-Phe, were treated with $diC_{18}Icc$ in the presence of 10 μM histamine. (×1650.)

presence of 10 μM histamine (control for the presence of Flu-Him above). In all cases, $diC_{18}Icc$ uniformly labeled the surface of PMNs. This indicates that the observed distribution of histamine receptors is not due to artifacts originating from membrane convolution but rather to a specific topographic rearrangement of the PMN cell surface in response to univalent binding of histamine.

DISCUSSION

The results of the present study are of technological and biological significance. Flu-Him is a fluorescent histamine-receptor probe that retains biological activity and receptor and pharmacological specificity. These conditions have generally not been fulfilled by previous histamine-receptor probes using protein carriers (1, 2).

The physiological role of the histamine ligand–receptor clusters is immediately suggested by their location. Punctate fluorescence is observed upon stationary cells. Cell locomotion leads to redistribution of fluorescence to pseudopods or to the lamellipodium and uropod. These cellular sites are responsible for adherence of PMNs and fibroblasts to substrates (23–27). Therefore, accumulation of histamine ligand–receptor complexes at these sites may sterically exclude membrane components participating in adhesion or decrease adhesion through the action of a second messenger such as cAMP. In either case, a structure–function correlation is suggested. Accumulation of histamine receptors at the leading edge of migrating PMNs may also increase cell sensitivity to an extracellular mediator such as histamine. Previous studies (28) have indicated that certain cell surface components undergo topographic reorganizations during cell movement, endocytosis, or "capping." The results of the present study are consistent with the Oliver–Berlin wave model of receptor redistribution (28). Moreover, our results clearly indicate that crosslinkage of receptors by multivalent ligands is not a requirement for cell surface responses fitting the Oliver–Berlin model, although receptor–receptor crosslinkage is not ruled out.

The fluorescent label described above will be applicable to the study of histamine receptors in their many diverse physiological settings, using microscopic and flow-cytometric techniques. The development of H1- and H2-specific fluorescent labels should allow simultaneous localization of these distinct receptors.

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