CELL REGULATION, Vol. 2, 41–55, January 1991

Internalization pathway of C3b receptors in human neutrophils and its transmodulation by chemoattractant receptors stimulation

Jean-Louis Carpentier,*† Daniel P. Lew,‡ Jean-Pierre Paccaud,* Roy Gil,* Barry Iacopetta,* Michel Kazatchkine,§ Olle Stendahl,‖ and Tullio Pozzan¶
*Institute of Histology and Embryology
University of Geneva Medical Center
1211 Geneva 4, Switzerland
‡Infectious Diseases Division
Department of Internal Medicine
University of Geneva
1211 Geneva 4, Switzerland
§Nephrology Division
Hôpital Broussais
Paris, France
‖Medical Microbiology Institute
University of Linköping, Sweden
¶Istituto di Patologia Generale
Ferrara, Italy

On the surface of phagocytes, C3b receptors (CR1) bind C3b-coated particles and promote their ingestion after activation by appropriate stimuli such as lymphokines or the chemoattractant formyl methionyl leucyl phenylalanine (fMLP) and fibronectin. The aims of the present study were 1) to define at the electron microscopic level the nature of the process responsible for CR1 internalization and 2) to dissect the mechanism by which a physiological activator (fMLP) stimulates this process. CR1 was visualized either by the immunogold technique or by quantitative electron microscopic autoradiography using a monoclonal anti-CR1 antibody. Both techniques revealed that after anti-CR1 binding, CR1 cluster on the neutrophil surface in a time-, temperature-, and antibody-dependent fashion, but do not concentrate in coated pits. CR1 internalization requires receptor cross-linking (does not occur in the presence of Fab fragments of anti-CR1) and intact microfilaments. It results in the association of the internalized material with large flattened vacuoles, organized in stacks. Together with the surface localization of CR1 close to cytoplasmic projections (ruffles), these observations suggest that uptake of CR1 occurs through a macropinocytotic process. Eventually, CR1 concentrate in lysosomal structures. fMLP markedly stimulates this pattern of CR1 internalization without affecting their clustering or their lack of association with coated pits. Stimulation by fMLP is inhibited by pertussis toxin, unaffected by preventing receptor-triggered cytosolic free calcium $[Ca^{2+}]_i$ elevations, and mimicked by phorbol myristate acetate. Taken together our data demonstrate 1) that, in neutrophils, CR1 is internalized via a coated pit independent macropinocytotic process, dependent on intact microfilaments and receptor cross-linking; 2) that, in the same cells, fMLP is internalized via the classical coated pits pathway; and 3) that fMLP amplifies CR1 uptake possibly via protein kinase C stimulation.

Introduction

Human neutrophils are highly mobile cells endowed with the capacity to adhere to various substrates and to sense, move towards (chemotaxis), ingest (phagocytosis), and kill invading microorganisms (Pozzan *et al.,* 1983; Malech and Gallin, 1987; Elsbach *et al.,* 1988; Stossel, 1988). These functions are mediated by several classes of receptors (adherence, chemoattractant, and phagocytic receptors) present on the surface of the cells. Chemoattractant receptors, which bind molecules such as C5a, formyl methionyl leucyl phenylalanine (fMLP), and LTB4, are coupled to phosphoinositide hydrolysis, which leads to an increase of cytosolic free calcium ($[Ca^{2+}]_i$) and activation of protein kinase C. In turn, this signaling cascade is thought to be essential for triggering oriented locomotion and secretion (DiVirgilio *et al.,* 1983; Korchak *et al.,* 1984; Lew *et al.,* 1984, 1986a,b; Sklar *et al.,* 1984; Andersson *et al.,* 1986; Snyderman *et al.,* 1986; Barrowman *et al.,* 1987). Among phagocytic receptors, those for Fc are endowed with the intrinsic ability to trigger ingestion and metabolic responses, whereas complement receptors (i.e., receptors for C3b and C3bi, named CR1 and CR3, respectively) promote phagocy-

† Corresponding author.

tosis only in presence of extrinsic molecules such as fMLP, and fibronectin or of pharmacological agents capable of activating protein kinase C such as phorbol myristate acetate (PMA) (Wright and Silverstein, 1982; Pommier *et al.,* 1984; Changelian *et al.,* 1985; Brown, 1986; Unkeless and Wright, 1988).

In the present study, we examined the mechanism by which CR1 is internalized and the effect of chemoattractant receptor activation on this process. CR1 was tagged by a mouse monoclonal anti-CR1 antibody or its Fab fragments, and its localization in human neutrophils over different times of incubation was determined by quantitative electron microscopic techniques. We provide direct evidence that, in these cells, upon CR1 cross-linking, this receptor is not internalized through the classical receptor-mediated endocytic pathway involving coated pits, but through a process requiring microfilament integrity and resulting in the formation of large intracellular vacuoles arranged in stacks. By contrast, we show that the chemotactic peptide fMLP is internalized through the coated pits pathway. These two different receptors internalized by different routes can cross-talk since fMLP stimulates CR1 internalization. This transmodulation possibly occurs through an activation of protein kinase C.

Results

Distribution of CR1 on the surface of neutrophils

Although some receptors (e.g., transferrin, low-density lipoprotein) are associated with coated pits even in the absence of ligands, others (e.g., insulin, epidermal growth factor) cluster in coated pits only after ligand binding. With respect to this second group of receptors, cross-linking with anti-receptor antibodies has been reported to mimick agonist binding. In the case of CR1, neither after 1 h of incubation with ^{125}I anti-CR1 at 4°C, nor after 7 or 19 min at 37°C did we find a detectable labeling of coated pits (Figures 1 and 2). By contrast, a time- and temperature-dependent labeling of coated pits was detected with ^{125}I formyl-nle-leu-nle-tyr-lys (^{125}I fNLNTP), an hexapeptide capable of binding and activating fMLP receptors and amenable to iodination (Figure 2).

The lack of association of CR1 with coated pits on the surface of neutrophils was verified using unlabeled anti-CR1 antibody followed, after prefixation, by an anti-IgG antibody coupled to colloidal gold (10 nm) (Figure 1): of 645 gold particles detected on thin sections of neutrophils (incubated for 1, 3, and 5 min at 37°C), only 4 were seen in coated pits. By contrast, both the autoradiographic and immunocytochemical techniques frequently localized labeled anti-CR1 close to thin cytoplasmic projections (ruffles) (Figure 1). An en face visualization of the surface labeling was made possible by the label fracture technique (Pinto de Silva and Kan, 1984; Carpentier *et al.,* 1985). Surface invaginations (most of them corresponding to coated pits and appearing as small elevated craters) remained unlabeled in all conditions studied (Figure 1). The label-fracture technique also allowed the evaluation of the aggregation state of CR1 on the surface of neutrophils. On cells incubated for 2 h at 4°C in the presence of anti-CR1, washed, prefixed, and further incubated with anti-IgG gold, most of the gold particles appeared single, but, progressively, they clustered as a function of incubation time at 37°C (Figure 1). Figure 3 shows a quantitation of the aggregation process as a function of incubation time and temperature. At 4°C, 60% of the gold particles were single, and clusters of >3 particles were rarely seen. Incubation of anti-CR1 antibody–labeled neutrophils at 37°C resulted in an increase in the percentage of particles in clusters of ≥3. No difference in the receptor-labeling pattern and clustering was observed after treatment with fMLP (10^{-6} M) (Figure 3). Clustering was not observed if cells were warmed at 37°C in the presence or absence of fMLP (10^{-6} M), prefixed, and further incubated with anti-CR1 antibody (data not shown).

An additional argument in favor of the internalization of CR1 through a pathway different from the classical receptor-mediated endocytosis involving coated pits was provided by the inhibition of ^{125}I anti-CR1 internalization by the microfilament-disrupting agent cytochalasin B (Figure 4). On the other hand, ^{125}I fNLNTP internalization, which occurs through coated pits, was unaffected by cytochalasin B (Figure 4).

Internalization of CR1 in neutrophils

To follow the fate of CR1 tagged with anti-CR1, neutrophils were incubated for 2 h at 4°C in the presence of ^{125}I anti-CR1, washed, and warmed for various periods of time at 37°C. As determined by quantitative electron microscopic (EM) autoradiography, ^{125}I anti-CR1 was progressively internalized and reached a maximum by ~10 min of incubation when 30% of the radioactive material was inside neutrophils (Figure 5). When CR1 was tagged with ^{125}I-Fab frag-

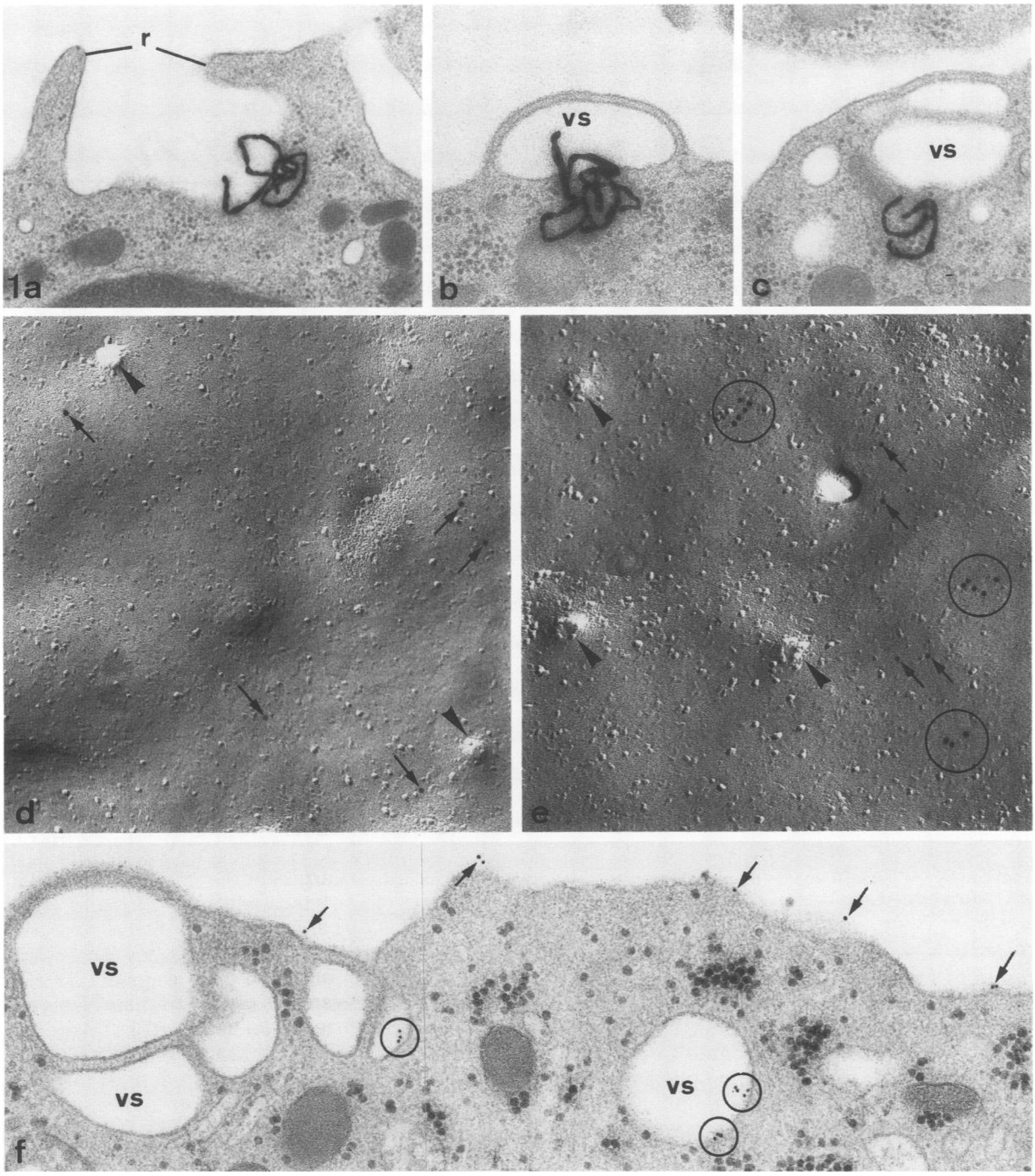

Figure 1. **Surface and early intracellular events during binding to human neutrophils of anti-CR1 antibody labeled with ^{125}Iodine (autoradiography, a–c) or anti IgG gold (label fracture, d and e, thin section f).** (a–c) Autoradiographic grains associated with a segment of the plasma membrane surrounded by ruffles (r) in a; with a newly formed vacuolar structure (vs) in b, or with vacuolar structures deeper inside the cytoplasm in c. 40 000×. (d and e) Label fracture replicas of the plasma membrane of neutrophils incubated in the presence of anti-CR1 antibody for 2 h at 4°C in d, for 2 h at 4°C, washed, and warmed at 37°C for 15 min in e. The anti-CR1 antibody was tagged with an anti-mouse IgG antibody coupled to colloidal gold (10 nm). Gold particles are mostly present as singlets at 4°C (arrows in d) and are frequently aggregated following the 37°C incubation (circles in e). Surface invaginations appear as small elevated craters (arrow heads). 83 000×. (f) View of a thin section of the peripheral cytoplasm of a neutrophil incubated as in e except that cells were warmed only for 5 min at 37°C. Gold particles mostly appear as singlets (arrows) on the cell surface and in small aggregates (circles) inside the cell. 57 000×.

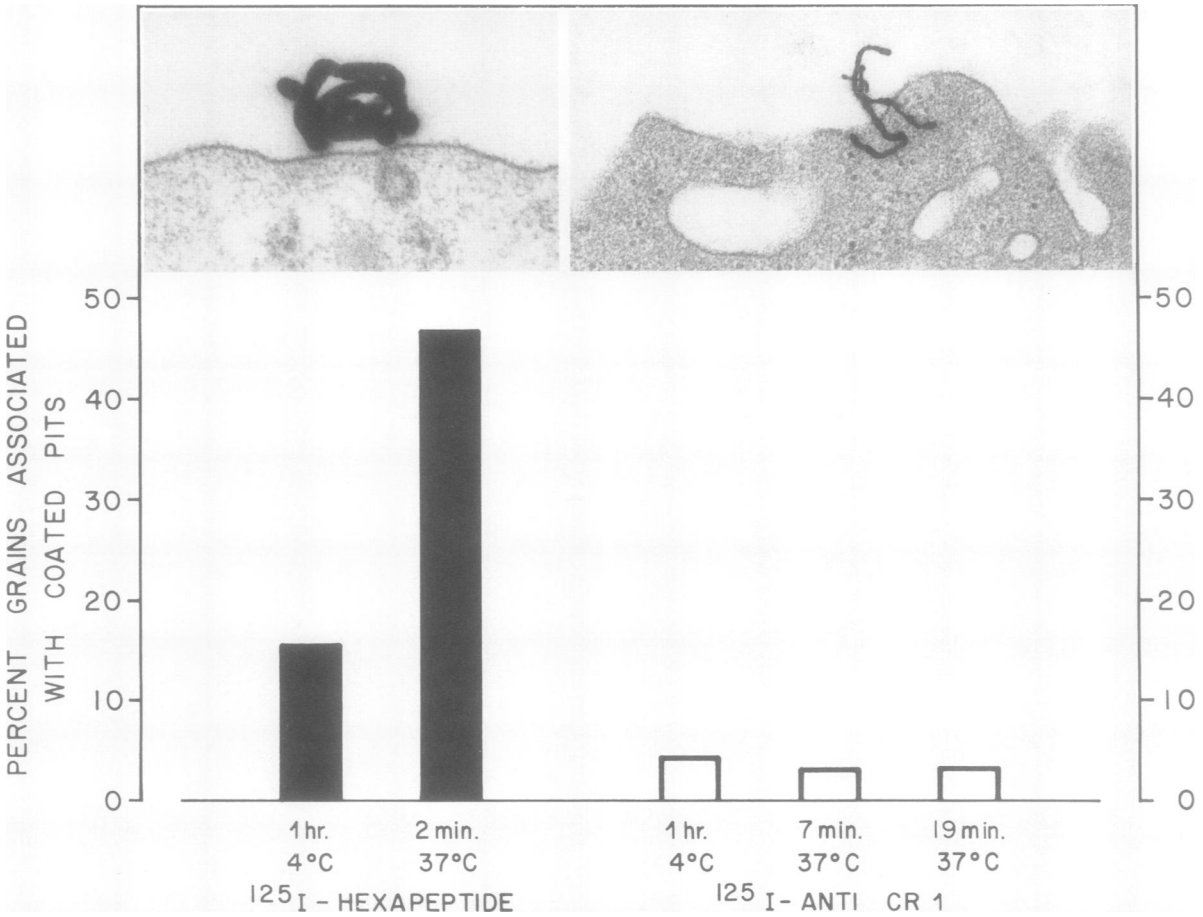

Figure 2. **Association of ^{125}I anti-CR1 (□) and ^{125}I fNLNTP (■) with coated pits on the surface of human neutrophils.** Results are expressed as percentage of the total number of grains associated with the cell surface (250 nm from the plasma membrane) in each experimental condition. Number of grains analyzed: ^{125}I fNLNPT: 1 h 4°C: 279; 2 min 37°C: 120; ^{125}I anti-CR1: 1 h 4°C: 320; 7 min 37°C: 170; 19 min 37°C: 324. The insets illustrate on the left a coated pit with an autoradiographic grain produced by ^{125}I fNLNTP and on the right a representative segment of membrane to which ^{125}I anti-CR1 is associated and which lacks coated pits.

ments of anti-CR1, there was no significative internalization of the receptor by 10 or 20 min of incubation at 37°C (Figure 5). By contrast, when neutrophils were incubated in the presence of C3b-coated immune complexes labeled with ^{125}Iodine, their internalization rate and pathway (see below) were superposable to the ones observed with ^{125}I anti-CR1 (data not shown), providing physiological relevance to the observations obtained with ^{125}I anti-CR1.

We have shown previously that the C3b-coated immune complexes used do not interact with Fc receptors since a monoclonal antibody directed against CR1 inhibits immune complexes binding, whereas an anti-Fc receptor antibody has no effect by itself and is not additive to anti-CR1 (Paccaud *et al.*, 1990). Moreover, we have determined biochemically that the internalization rate of ^{125}I-F(ab)$_2$ fragments of our anti-CR1 antibody was similar to that observed with intact ^{125}I anti-CR1 (Figure 6). Taken together with the fact that murine IgG$_1$ poorly (if at all) binds to Fc receptors present at the surface of human neutrophils (as verified in our system with a mouse IgG$_1$ kappa monoclonal antibody, which does not bind to human neutrophils), and the fact that we are applying tracer amounts of ^{125}I anti-CR1, these observations exclude the possibility of anti-CR1 internalization through its Fc portion.

Internalization pathway of CR1 in neutrophils

To determine which intracellular structures are involved in CR1 internalization, we evaluated the percentage of ^{125}I anti-CR1 associated with the

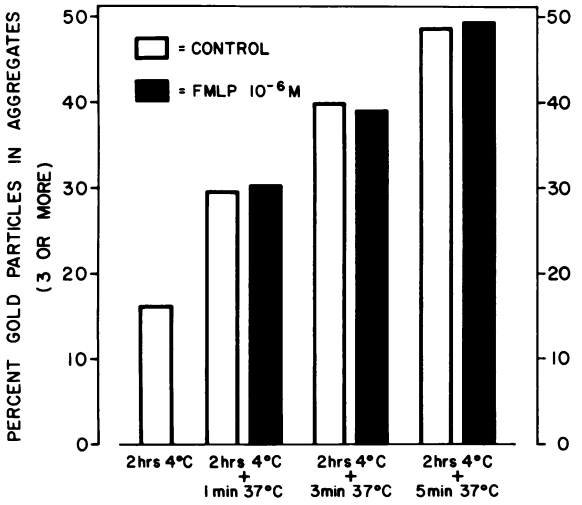

Figure 3. **Aggregation of CR1 at the surface of neutrophils.** Cells were incubated with anti CR1 for 2 h at 4°C, washed, and further incubated for various periods of time at 37°C in the presence (■) or absence (□) of fMLP 10^{-6} M. Surface-bound anti-CR1 were tagged with anti-mouse IgG coupled to colloidal gold and the percentage of gold particles in clusters of ≥3 was calculated as percent of the total number of gold particles counted (~1000 per condition). This histogram is representative of the results obtained in three different experiments.

various intracellular compartments at the different incubation times at 4°C and 37°C (see Materials and methods). These values are representative of the labeling changes inside these compartments because the relative cytoplasmic volumes occupied by these structures remained practically unchanged during the whole incubation period (Table 1). After a 2-min incubation at 37°C, 50% of the autoradiographic grains overlying the cytoplasm were associated with large vacuoles, frequently organized in stacks (Figures 7 and 8). The most peripheral vacuoles showed continuity with the cell surface but the deeply located cisternae were clearly disconnected. Indeed, after a 2-min incubation at 37°C with horseradish peroxidase (HRP), followed by extensive washings of the cells at 4°C and fixation, HRP was washed out of the peripheral vacuoles but remained trapped inside the inner ones (Figure 8). These cisternae form stacks with numerous bulging, coated vesicles (Figure 8). These structures present a general organization similar to that of a Golgi complex, but they are clearly distinct from this organelle, poorly developed in neutrophils, and more centrally located (Figure 7). The association of the internalized ^{125}I anti-CR1 with the above-described vacuoles and cisternae declined with incubation time (Figure 9) and the radioactivity was progressively recovered in multivesicular bodies and other lysosomal structures (Figures 7 and 9). A similar sequence of events was observed with anti-CR1 tagged with anti-IgG-gold (Figure 7) (quantitative data not shown).

Effect of fMLP on CR1 internalization

Addition of fMLP by 5 min of incubation at 37°C resulted in a marked increase of ^{125}I anti-CR1 internalization (Figures 5a and b and 6). PMA (50 nM), which is thought to specifically activate protein kinase C, stimulated the internalization of ^{125}I anti-CR1 to a similar extent (Figure 10).

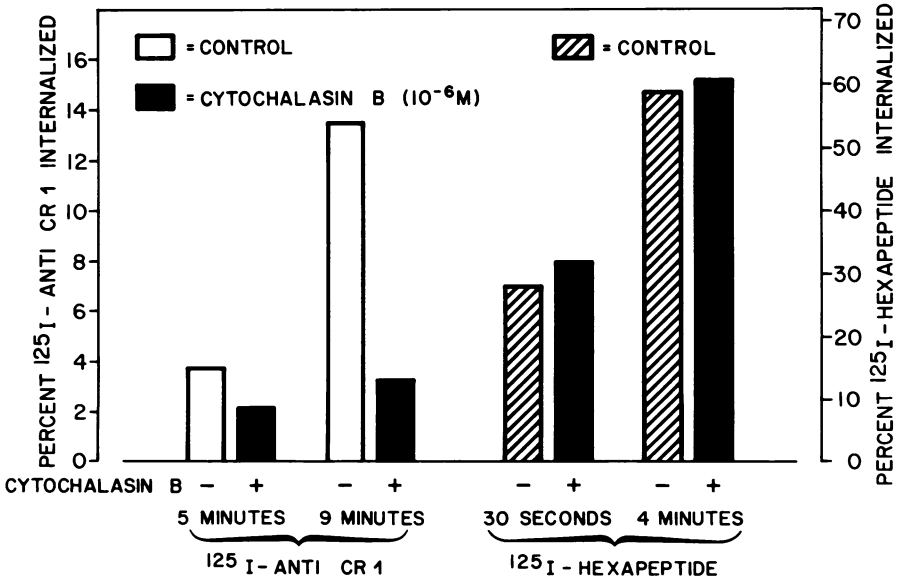

Figure 4. **Effect of cytochalasin B (10^{-6} M) on ^{125}I anti-CR1 and ^{125}I fNLNPT internalization in neutrophils.** ^{125}I fNLNPT being internalized much more rapidly than ^{125}I anti-CR1, cytochalasin B was added 5 min before starting the incubation with ^{125}I fNLNPT at 37°C and the incubation times were shorter than in the case of anti-CR1. Results are the mean of two different experiments.

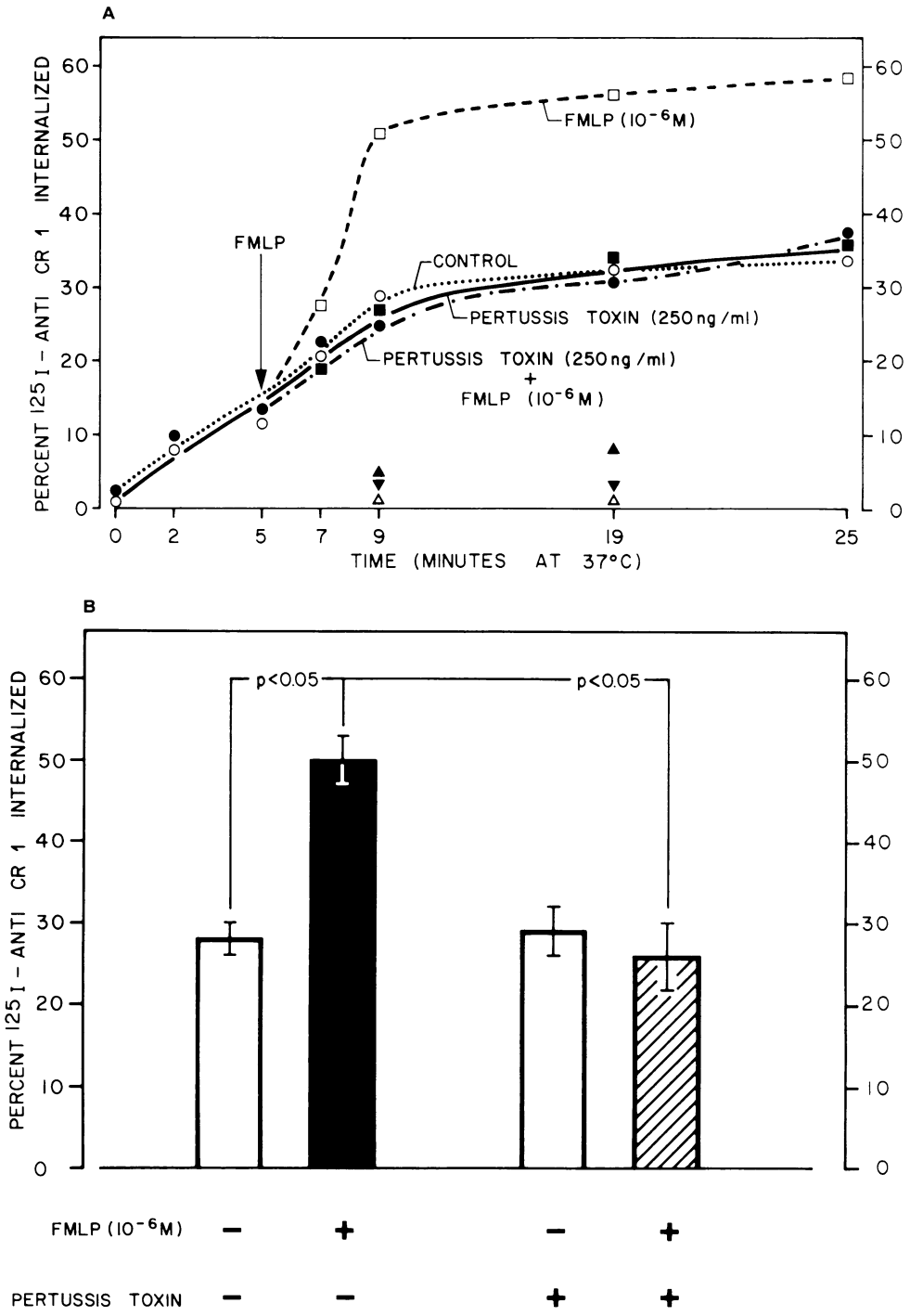

Figure 5. **Effect of fMLP in the presence or absence of pertussis toxin on the internalization of ^{125}I anti-CR1 antibody in human neutrophils.** Cells suspended in calcium medium were preincubated with 250 ng/ml pertussis toxin (●, ■) or with medium alone (○, □) for 2 h at 37°C. After washing, the cells were further incubated for 1 h at 4°C with ^{125}I anti-CR1 antibody. Following three washings the cells were incubated at 37°C for various periods of time. When indicated, fMLP (10^{-6} M) was added to the incubation medium (□, ■). The internalization of the radioactive ligand was determined by quantitative EM autoradiography. Autoradiographic grains were considered to localize internalized material if their center was >250 nm beyond the plasma membrane. They were expressed in percent of the total number of grains. Open triangles illustrate the internalization of ^{125}I-Fab fragments of the antibody. Filled triangles illustrate the influence of fMLP (▲) or fMLP + pertussis toxin (▼) on the internalization of ^{125}I Fab fragments of anti-CR1. (A) The pattern of a typical experiment. (B) The results of a statistical evaluation carried out on three different experiments after 19 min of incubation at 37°C.

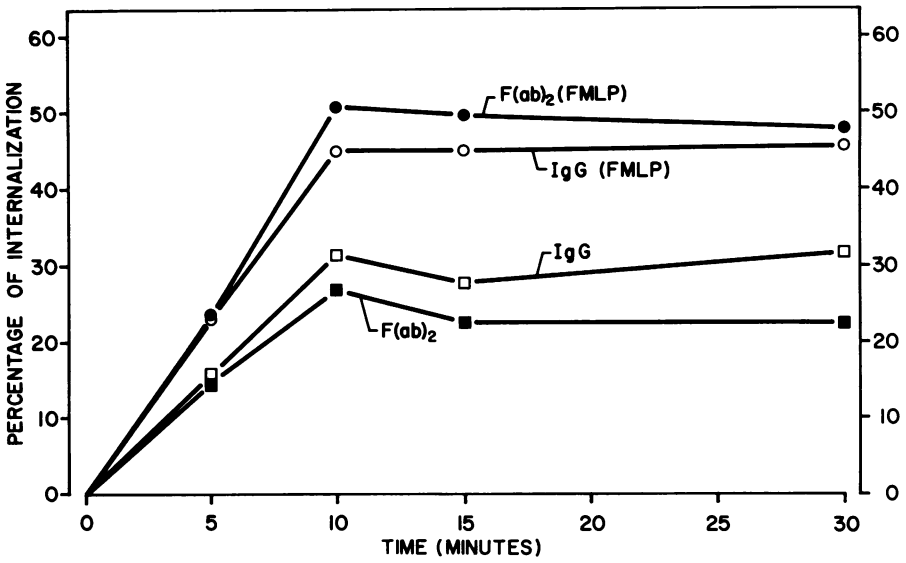

Figure 6. **Comparison of the internalization rate of anti-CR1 as $F(ab)_2$ fragments and whole IgG.** PMN were incubated 2 h in the presence of labeled J3D3 [$F(ab)_2$ fragments (●, ■), IgG (○, □)], washed, and warmed for various period of time at 37°C in the presence (○, ●) or absence (□, ■) of fMLP (10^{-6} M). Internalization was measured after trypsinization to remove cell surface associated material. Data are representative of three different experiments.

To investigate whether the stimulatory effect of fMLP depended on the activation of the signaling cascade triggered by this agonist, neutrophils were preincubated with pertussis toxin (250 ng/ml) for 2 h. By ADP-ribosylating a specific G regulatory protein, pertussis toxin blocks signal transduction without affecting fMLP binding (Brandt *et al.*, 1985; Sklar *et al.*, 1984). Figure 5, A and B, shows that the stimulatory effect of fMLP on CR1-antibody complexes internalization was completely abolished. By contrast, the basal rates of ^{125}I anti-CR1 internalization and the stimulatory effect of PMA were unaffected by pertussis toxin (Figure 5, A and B, and 10).

When CR1 was tagged with ^{125}I-Fab fragments of anti-CR1, fMLP had only a weak stimulatory effect on the internalization of the receptor which was abolished by pertussis toxin (Figure 5).

Table 1. Volume densities* of various intracellular structures in neutrophils after 2–25 min incubation at 37°C in the presence of anti-CR1†

	2 min (n = 46)‡	25 min (n = 50)‡
Granules	20.4 ± 1.1	19.2 ± 0.9
Vacuoles	1.7 ± 0.5	1.1 ± 0.2
Multivesicular bodies	2.8 ± 0.4	4.1 ± 0.4

* Volume density = V_V according to Weibel (1969).

† Values are means ± SEM.

‡ n = number of cell sections analyzed.

Hydrolysis of phosphoinositols caused by fMLP results in the formation of two second messengers, diacylglycerol (DAG), which activates protein kinase C, and $Ins(1,4,5)P_3$, which mobilizes intracellular Ca^{2+}. To distinguish whether increased CR1 internalization was related to the rise in $[Ca^{2+}]_i$ or possibly by activation of protein kinase C by DAG, fMLP was added under conditions known to prevent agonist-dependent $[Ca^{2+}]_i$ rises [pretreatment with the Ca^{2+} ionophore ionomycin in Ca^{2+}-free medium containing 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (Pozzan *et al.*, 1983; Lew *et al.*, 1984, 1986a,b)]; under these conditions, protein kinase C-dependent functions are still activable (DiVirgilio *et al.*, 1985; Pozzan *et al.*, 1983; Lew *et al.*, 1984, 1986a,b). Figure 11 shows that the stimulation of CR1 internalization by fMLP occurred even in the absence of $[Ca^{2+}]_i$ rises. Taken together, these results suggest that the stimulation by fMLP of CR1 internalization is mediated through the activation of protein kinase C, secondary to phosphoinositide hydrolysis and DAG formation.

Discussion

The results described here argue that, in neutrophils, a basic difference exists between the internalization of CR1, which does not appear to involve coated pits, and the uptake of fMLP receptors, which occurs through the classical coated pits route followed by most polypeptide hormone, growth factor, and transport protein receptors. This is in contrast to a previous report

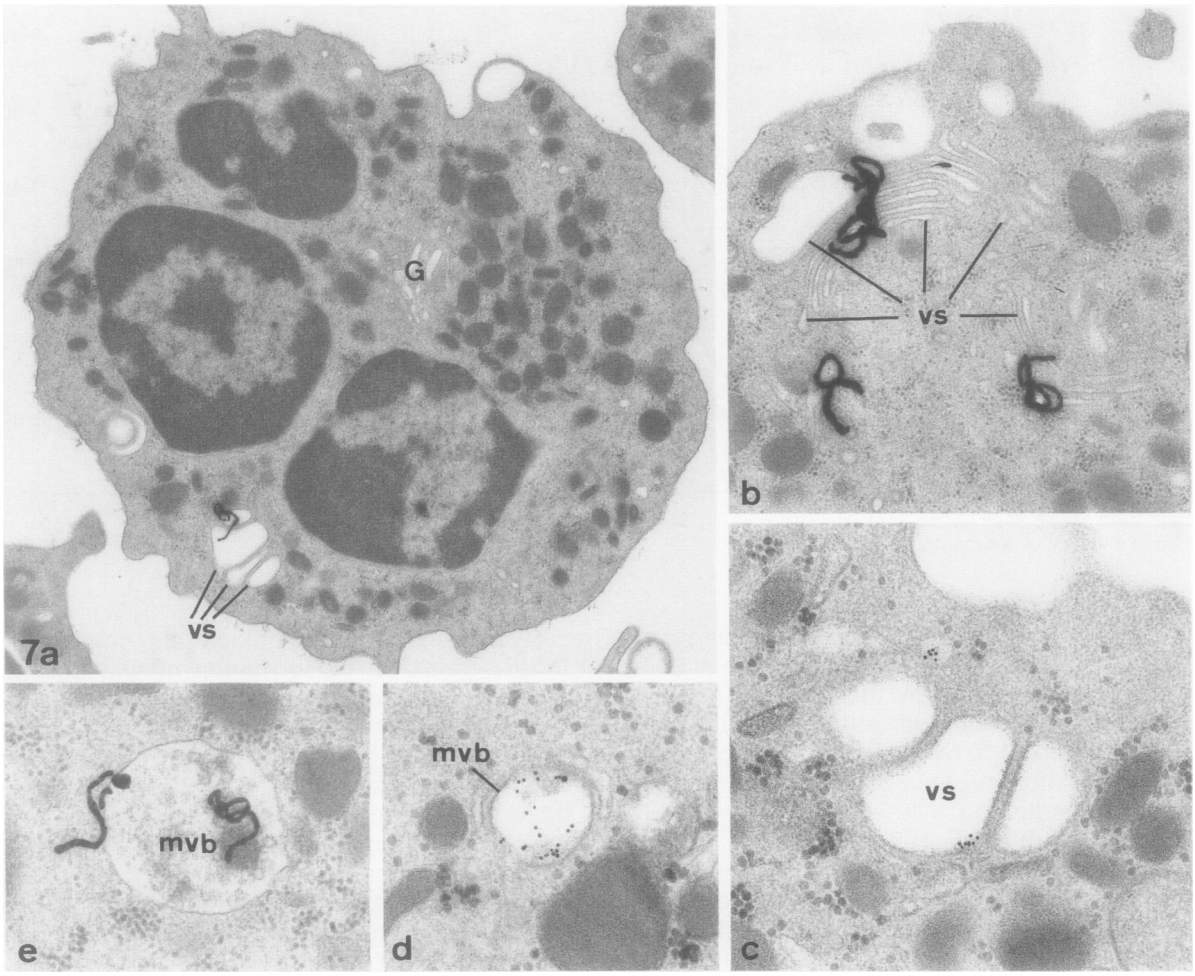

Figure 7. **Intracellular localization of anti-CR1 coupled to ^{125}Iodine or IgG gold in neutrophils.** The antibody either tagged with the radioactive isotope (a, b, and e) or with colloidal gold (c and d) is recovered first in large vacuolar structures (vs) frequently organized in stacks (a–c) and later in multivesicular bodies (mvb) (d and e); G: Golgi complex. a: 16 000×; b: 22 000×; c: 51 000×; d: 59 000×; e: 29 000×.

(Abrahamson and Fearon, 1983) where the internalization of CR1 in neutrophils was described to be preceded by its association with coated pits. Our observation is, however, similar to that of Detmers *et al.* (1987) for CR3, the other complement receptor involved in phagocytosis, which was not seen associated with coated pits on the surface of neutrophils. In addition to quantitative morphological observations supporting this conclusion, key evidence for such a concept is that CR1 internalization is severely curtailed by the microfilament-disrupting drug cytochalasin B (O'Shea *et al.,* 1985; Jack *et al.,* 1986, and the present report), which does not disturb coated pits endocytosis (Wills *et al.,* 1972; Terris *et al.,* 1979; Salisbury and Keller, 1983, and present data). Thus it would appear that in neutrophils, CR1 is internalized by a macropinocytotic process resembling phagocytosis. As in phagocytosis, this process presumably requires the development of cytoplasmic projections or ruffles, whose formation would be sensitive to cytochalasin B. Previous studies carried out at the optical level suggested that, instead of forming phagosomes, macropinocytosis leads to the formation of large intracellular vacuoles (Lewis, 1931; Willingham and Yamada, 1978). This is what we demonstrated at the EM level: inside the cells, ^{125}I anti-CR1 indeed accumulates into large vacuoles but, in addition, we found the internalized ^{125}I anti-CR1 closely associated with stacked cisternae, showing a general organization similar to the one of the Golgi complexes. We showed, however, that this compartment is clearly distinct from the Golgi complex. Our ^{125}I-fMLP an-

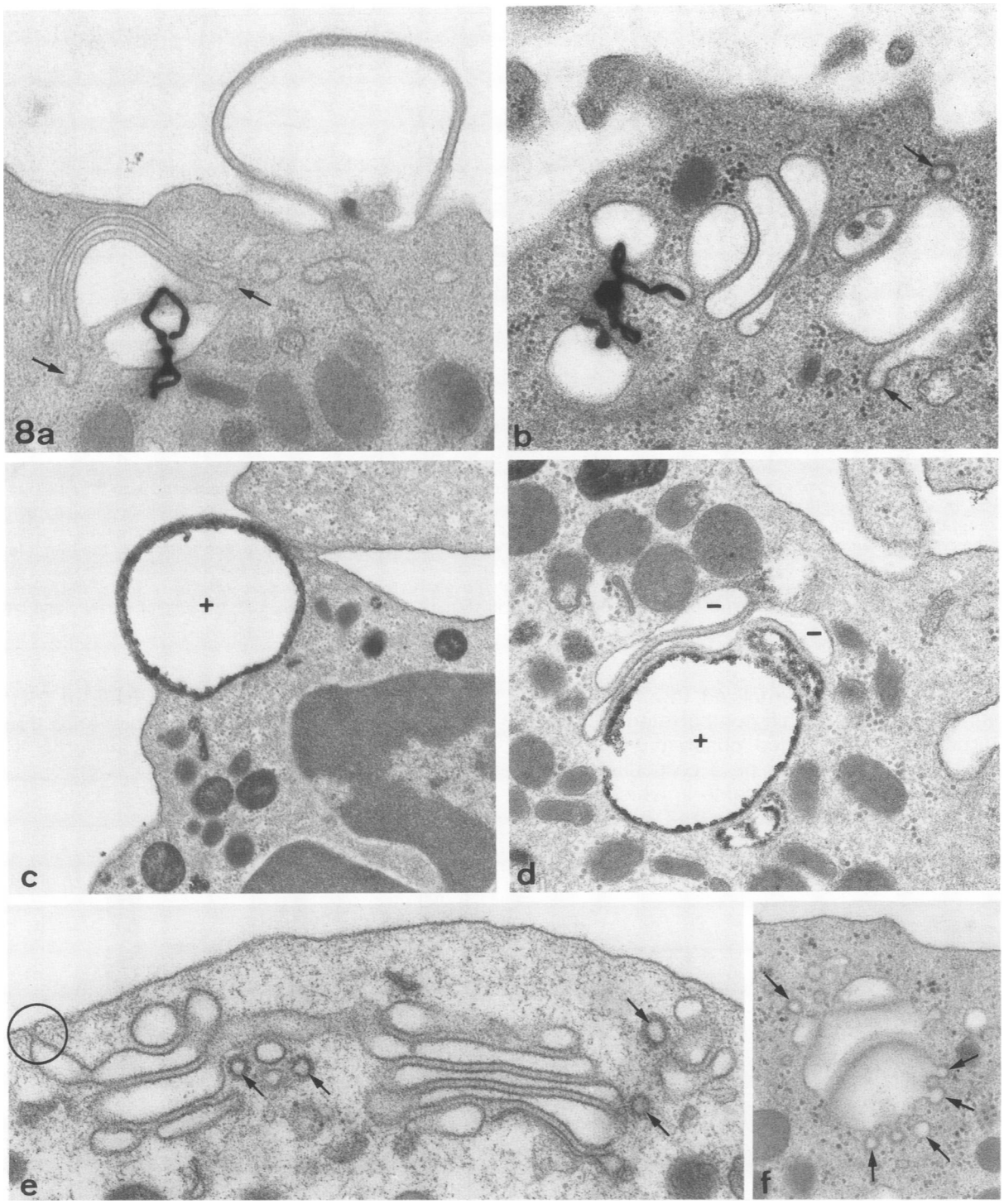

Figure 8. **Representative examples of the morphology of vacuolar structures.** These structures, with which autoradiographic grains localizing ^{125}I anti-CR1 are associated, appear as large vacuoles or flattened cisternae organized in stacks (a and b). After a 2-min incubation with HRP followed by extensive washings, some vacuoles appear free of HRP labeling, whereas others contain the marker (c and d). This confirms their endocytotic function and suggests that the empty vacuoles maintain a connection with the cell surface allowing the washing away of the marker. In appropriate plane of sections, the opening of the peripheral cisternae to the cell surface can be seen (e). Numerous coated vesicles (arrows) are seen in the vicinity of or budding from these structures (a, b, e, and f). a: 32 000×; b: 27 000×; c: 41 000×; d: 32 000×; e: 41 000×; f: 32 000×.

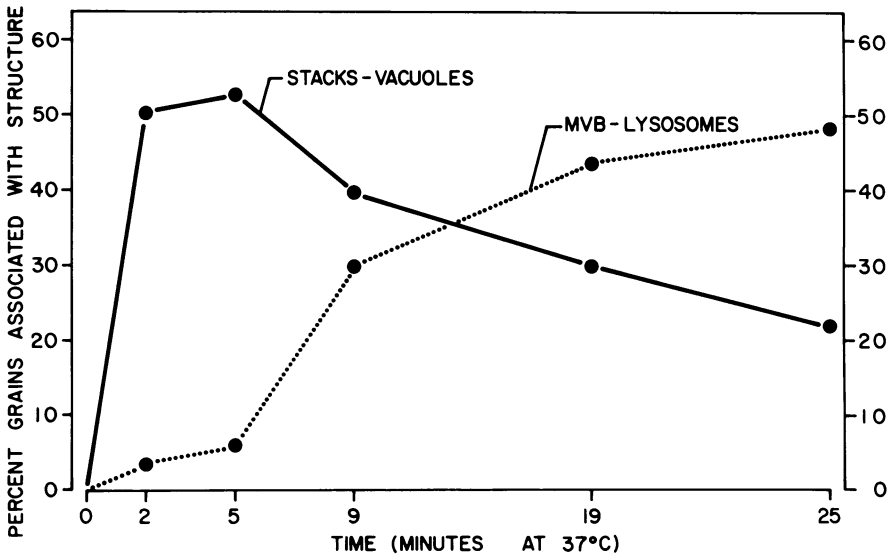

Figure 9. **Relationship of autoradiographic grains with intracellular structures in neutrophils incubated with ^{125}I anti-CR1.** Results are expressed in percent autoradiographic grains localizing internalized ^{125}I anti-CR1 (250 nm from the plasma membrane) that associate with stacks and vacuoles, multivesicular bodies, and lysosomes or with granules (the total of these three compartments is considered =100%). The association with the granules was not illustrated to simplify the graph [at no one time point did we find a preferential association of the grains (ratio of percent grains associated with granules over relative cytoplasmic volume occupied by granules above 1) with these structures]. Results presented are a pool of the results obtained from three individual incubations. Number of grains analyzed: 2 min: 109; 5 min: 140; 9 min: 314; 19 min: 304; 25 min: 286.

alogue, although internalized through coated pits, also ended in this compartment (Gil and Carpentier, unpublished observation); hence, these would appear to be a particularly well-developed form of endosome in which ligand-receptor complexes internalized by different routes all converge before being sorted to various intracellular destinations (Helenius *et al.*, 1983; Tran *et al.*, 1987). In the case of CR1, most of the internalized receptors appeared eventually in multivesicular bodies and other lysosomal structures.

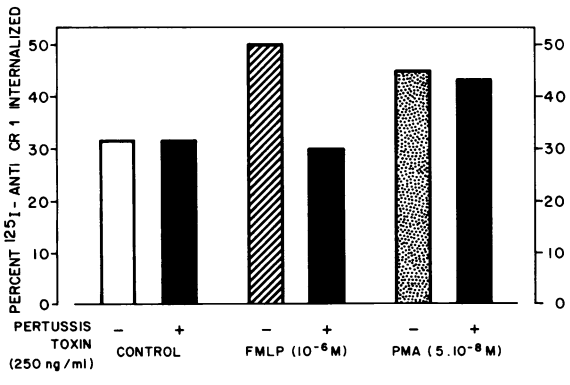

Figure 10. **Comparison of the effect of fMLP (10^{-6} M) and PMA (5×10^{-8} M) on ^{125}I anti-CR1 internalization in the presence or absence of pertussis toxin (250 ng/ml).** Incubation was carried out for 19 min at 37°C. The experimental protocol was identical to that described in Figure 5. Results presented are representative of those obtained in three different experiments.

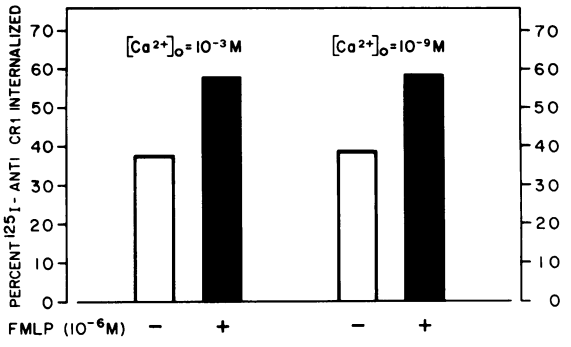

Figure 11. **The $[Ca^{2+}]_i$ elevation triggered by fMLP receptor activation is not necessary for the stimulation of ^{125}I anti-CR1 antibody internalization.** The cells were warmed for 4 min either in calcium medium (left), $[Ca^{2+}]_i = 10^{-3}$ M, [this treatment empties intracellular Ca^{2+} stores and prevents fMLP induced $[Ca^{2+}]_i$ elevation (Korchak *et al.*, 1984; Lew *et al.*, 1984; 1986a; Pommier *et al.*, 1984)] or in calcium free medium + EGTA (right), $[Ca^{2+}]_i = 10^{-9}$ M. Ionomycin was added 1 min before fMLP in the cells previously incubated in Ca^{2+} free medium + EGTA (see also Materials and methods). Cells were further incubated for 14 min at 37°C. These results are representative of those obtained in three different experiments. Similar results were obtained at two different time points (4 and 20 min).

The internalization of anti-CR1 by macropinocytosis is receptor-mediated and specific. Indeed, neutrophils incubated with ^{125}I anti-CR1 plus an excess of unlabeled anti-CR1 bind and internalize only a very small amount of radioactivity. In addition, by contrast to intact anti-CR1, Fab fragments of anti-CR1 antibody are poorly internalized, indicating also that CR1 cross-linking by anti-CR1 antibody (confirmed by the CR1 aggregation quantitated on label-fracture replicas) is a key step in the internalization of the receptor in our experimental conditions. Such experimentally induced CR1 cross-linking could mimick CR1 clustering induced by physiological ligands such as immune complexes covered with C3b, which are internalized at the same rate as anti-CR1 antibodies. According to such a model, ligand-induced CR1 aggregation would be one of the first necessary steps in the sequence of events leading to CR1 internalization by macropinocytosis.

CR1 internalization process can be stimulated by the chemotactic peptide fMLP as well as by the pharmacological agent PMA (Changelian *et al.,* 1985; Turner *et al.,* 1988, and present data). Data presented here allowed us to dissect not only the pathway of CR1 internalization but also to analyze the signal necessary to enhance the process upon chemoattractant receptor activation. They suggest that activation of protein kinase C by endogenously produced DAG or other stimulatory signal(s) triggered by fMLP is what up-modulates CR1 endocytosis. Indeed, the inhibitory effect of pertussis toxin demonstrates that the stimulation is due to second messenger generation, whereas the lack of inhibition by blocking receptor-triggered calcium elevation indicates that a $[Ca^{2+}]_i$ increase is not involved in stimulating CR1 internalization. Moreover, our data support the concept that the increase in CR1 internalization does not result simply from a general stimulation of plasma membrane uptake, but rather involves alterations in CR1 itself, which is taken up selectively. We favor this concept for the following reasons: 1) In contrast to ^{125}I anti-CR1, the internalization of another surface receptor [^{125}I fNLNTP at a concentration (10^{-7} M) increasing phosphoinositide hydrolysis] was not inhibited by pertussis toxin (Gil and Carpentier, unpublished observation); 2) fMLP has a much weaker stimulatory effect on the internalization of ^{125}I-Fab anti-CR1 fragments than on the internalization of intact ^{125}I anti-CR1; and 3) fMLP and PMA increased CR1 internalization to a same extent (see Figure 9), whereas the formation of intracellular vacuoles is increased by a factor of 10 by PMA but by <2 by fMLP (data not shown, and Robinson *et al.,* 1987).

The stimulatory effect of fMLP and PMA cannot be attributed to a change in CR1 aggregation since CR1 does not spontaneously cluster in neutrophils pretreated with either activator of protein kinase C before prefixation and tagging with anti-CR1 antibody revealed by anti-IgG gold (present data and manuscript in preparation). Thus protein kinase C activation seems to stimulate CR1 internalization by a different mechanism or at a different step than anti-CR1 antibody. Such a selective stimulation of the internalization of specific membrane proteins could occur via changes in location or affinity for the uptake mechanism. Such phenomenon does occur during coated vesicle uptake of several different membrane receptors, namely, those that become phosphorylated in response to protein kinase C (Jacobs *et al.,* 1983; Cochet *et al.,* 1984; May *et al.,* 1984; Changelian and Fearon, 1986). Indeed, CR1 phosphorylation is also induced by fMLP or PMA presumably via activation of protein kinase C (Changelian and Fearon, 1986). Hence, phosphorylation could be a critical change that would somehow increase the likelihood of CR1-receptor incorporation into vacuoles formed via plasma membrane ruffling in neutrophils.

In conclusion, our results demonstrate that, in the presence of anti-CR1 antibody, CR1 is selectively endocytosed by neutrophils via a macropinocytotic process independent of coated pits and resembling phagocytosis in several aspects, in particular its requirement for intact microfilaments. The process of CR1 internalization is enhanced by the activation of another class of receptors (receptors for fMLP) via stimulation of protein kinase C or another excitatory signal independent of $[Ca^{2+}]_i$. Such cross-talking between chemoattractant receptors and phagocytosis receptors might be of utmost physiological importance in vivo at the inflammatory sites, where neutrophils ready for ingestion are stimulated by high concentrations of chemoattractants.

Methods

Isolation of neutrophils

Neutrophils from normal donors were isolated at room temperature from citrate-treated blood by dextran sedimentation, Ficoll-Hypaque cushions, and hypotonic lysis as previously described (Barrowman *et al.,* 1987).

Reagents and buffers

fMLP, PMA, cytochalasin B, ionomycin, horseradish peroxydase type II, poly-L-lysine, and N-formyl-nle-leu-phe-nle-

tyr-lys (fMLP hexapeptide analogue) were supplied by Sigma Chemical Co., St. Louis, MO. N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide-hydrochlorid was purchased from Fluka Chemie AG, Buchs, Switzerland and pertussis toxin was from List Biological Laboratories Inc. (Campbell, CO). The cells were incubated in a medium containing 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 μM EGTA, 1 mM NaH_2PO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4. This medium will be referred to as "calcium medium"; calcium-free medium will refer to this medium without $CaCl_2$ but supplemented with 1 mM EGTA, i.e., $[Ca^{2+}]_o = 10^{-9}$ M.

Antibodies

Monoclonal anti-CR1 antibody (J3D3) was obtained and iodinated by the Iodogen procedure as previously described (Brown, 1986). $F(ab)_2$ fragments of J3D3 were produced by pepsin digestion, and Fab fragments by digestion, followed by reduction and alkylation as previously described (Parham, 1986). The fragments were purified by FPLC (Pharmacia, Sweden) liquid chromatography on Superose 12 so as to obtain a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Goat anti-mouse IgG coupled to colloidal gold (10 nm) was obtained from Janssen Life Sciences Products (Beerse, Belgium). As determined by computerized morphometric image analysis, 93% of the gold particles appeared as singlets.

Internalization of $F(ab)_2$ fragments

PMV were incubated for 2 h at 4°C in the presence of excess labeled $F(ab)_2$, as whole IgG of J3D3 mAb, and washed twice. The cells were then warmed for various periods of time at 37°C and the incubation was stopped by adding 5 volumes of cold phosphate-buffered saline (PBS). Surface bound material was removed by brief treatment (3 min) with 0.5 mg/ml trypsine at 37°C, cell-bound, and supernatant was assessed for radioactivity.

Incubation for electron microscopic autoradiography

Purified neutrophils were divided into two batches at a final cell concentration of 10^7/ml. The first batch was treated with 250 ng/ml pertussis toxin for 2 h at 37°C, whereas the second (control) was left in buffer alone. After this incubation, the cells were washed twice and resuspended at a concentration of 10^7 cells/ml at 4°C for 1 h in the same medium supplemented with 2% heat inactivated human serum and ^{125}I anti-CR1 antibody or ^{125}I-Fab fragments of anti-CR1 ($=2 \times 10^5$ cpm). The cells were washed twice with ice-cold buffer, warmed for 5 min at 37°C before subsequent incubation in the presence or absence of fMLP (10^{-6} M) or PMA (5×10^{-8} M) for various periods of time at 37°C. In experiments designed to test the role of cytosolic free calcium, neutrophils incubated in the presence of the radiolabeled antibody were washed twice, as described above, before being resuspended in the same buffer without added Ca^{2+} and with 1 mM EGTA (extracellular free calcium concentration = 10^{-9} M). Cells were then warmed for 4 min at 37°C before adding the calcium ionophore ionomycin (10^{-6} M). One minute later, the cells were stimulated with fMLP (10^{-6} M). Controls were treated as described but without fMLP or PMA. In experiments where cytochalasin B was used, the drug (10^{-6} M) was added just before the incubation at 37°C.

At the indicated times, aliquots from the different experimental conditions were withdrawn and centrifuged at 200 $\times$ *g* for 5 min. The cell pellet was fixed with 2% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for a minimum of 2 h.

Quantitative electron microscopic autoradiography

After fixation with glutaraldehyde, cells were postfixed in osmium tetroxide and processed for quantitative electron microscopic autoradiography as previously described (Carpentier *et al.*, 1978). Thin sections were examined in a Philips EM 301 electron microscope (Philips, Eindhoven, The Netherlands) and autoradiographic grains were quantitatively analyzed at a magnification of 12 000× on cells that were judged to be well preserved.

For each incubation time in each experiment, three different Epon blocks were prepared and three sections were cut from each block. Thus for each time point in each experiment, nine separate grids were examined, out of which 300–400 autoradiographic grains (associated with 200–300 sections of different cells) were analyzed. A minimum of three different experiments were performed for each condition.

The association of the labeled material with the plasma membrane of neutrophils was assessed quantitatively as follows: a transparent plastic sheet on which a 250-nm circle had been drawn was superimposed on each autoradiographic grain; the grain was considered associated with the plasma membrane if a segment of the latter was contained within the circle. The number of such grains was then expressed as percent of the total number of grains counted. Percent grains internalized was calculated as the percent of total number of grains which were not associated with the plasma membrane. Grains associated with the plasma membrane were further separated according to the morphology of the underlying segment: 1) coated pits, 2) noncoated segments, and 3) unattributable. Grains were considered associated with coated pits if the grain center was within 250 nm from this specialized segment of the cell surface. They were categorized as unattributable when the exact morphology of the plasma membrane could not be unequivocally identified. This approach has been extensively used and validated in previous studies (Carpentier *et al.*, 1981, 1982, 1985).

The relationship of autoradiographic grains to intracellular structures was determined by superimposing to each autoradiographic grain the 250-nm diameter circle. Grains overlying the cytoplasm (>250 nm from the plasma membrane) were divided into the following classes based on their relation to the following structures: 1) large vacuoles and cisternae, 2) azurophilic or specific granules, and 3) lysosomal structures (including multivesicular bodies). The data were quantitated as previously described (Carpentier *et al.*, 1979, 1986).

Morphometry

The volume density (Vv) of the above-mentioned cytoplasmic structures were determined on thin sections by the point counting method (Weibel, 1969). Morphometric determinations were made on photographic prints enlarged three times (final magnification 36 000×) with a test screen (160 $\times$ 205 mm) in the form of a double-square lattice (with a ratio of 1:9). The rectangular frame of the electron micrograph enclosed 891 test points, of which 99 were set off as heavy points. The coarse grid was useful to estimate the volume of the cytoplasm. The fine grid was used to estimate the volume of the various structures mentioned above. The volume density of each structure considered was then ex-

pressed in percent of the total volume density of these structures. The determinations were carried out under two different incubation conditions: 2 min and 19 min of incubation at 37°C after a 2-h incubation at 4°C in the presence of ^{125}I anti-CR1 antibody. For each of these conditions a total of 48 pictures were analyzed. Random sampling was ensured by photographing in the electron microscope the closest neutrophil cytoplasm to each corner of 12 consecutive squares of the supporting grid. Pictures were taken at an initial magnification of 12 000×.

Incubation with HRP

Neutrophils were incubated for 2, 5, or 15 min at 37°C in the presence of 5 mg/ml of horseradish peroxidase in the presence or absence of fMLP (10^{-7} M). At the end of the incubation, cells were washed twice in 10 ml of calcium medium at 4°C before being fixed in glutaraldehyde 2.5% in cacodylate buffer 0.1 M (pH 7.4). Cells were reacted with diaminobenzidine and oxygene peroxyde according to Graham and Karnovsky (1966).

Incubation for immunoelectron microscopy

Freshly purified neutrophils (2×10^7) were first incubated for 2 h at 4°C with anti-CR1 antibody (diluted 1/10). Cells were then centrifuged ($200 \times g$) for 5 min at 4°C and resuspended in PBS supplemented with 1% fetal calf serum (FCS). This washing procedure was repeated twice. Cells were then either prefixed (see below) or further incubated in the absence of antibody for various periods of time at 37°C in the presence or absence of fMLP (10^{-6} M) before prefixation. Prefixation was carried out as proposed by Detmers *et al.* (1987) using a mixture of 1% ethyldimethylaminopropyl carbodiimide and 0.2% glutaraldehyde for 7 min at room temperature. After centrifugation for 5 min at $200 \times g$ at 4°C, cells were incubated for 15 min in ice-cold 0.5 mg/ml $NaBH_4$ and then washed three times with ice-cold medium. The cell pellet was finally resuspended in PBS supplemented with 1% FCS. Cells were then exposed for 2 h at 4°C to goat anti-mouse IgG coupled to 10 nm colloidal gold particles (diluted 1/10 in PBS + 1% FCS). After two washings (see above) neutrophils were fixed for 30 min at room temperature with 2.5% glutaraldehyde in cacodylate buffer (0.1 M pH 7.4). Cells were then either sedimented on plastic coverslips (10 mm) precoated with 1% Poly-L-Lysine for 15 min at room temperature for freeze-fracturing or postfixed in osmium tetroxide and dehydrated for conventional thin sections. Control incubations included omission of the first antibody, replacement of the first antibody with normal mouse IgG, and addition of the anti-CR1 antibody after the prefixation following a 4°C incubation. While in the first two conditions we did not see any significative labeling, in the third condition the labeling pattern was similar to that observed when the anti-CR1 antibody was added at 4°C before prefixation.

Freeze-fracture

Fixed, labeled PMN adherent to plastic coverslips were impregnated for 2 h in 30% (vol/vol) glycerol in 0.1 M cacodylate buffer, pH 7.4; the coverslips were cut into 1.5×0.5 mm squares, inverted on specimen carriers (Balzers, Liechtenstein), and freeze-fractured at −110°C in a Balzers BAF 301 apparatus according to Pauli *et al.* (1977). Fractured faces were replicated by platinum/carbon evaporation. As described by Pinto da Silva and Kan (1984), the replicas were washed by successive floatings on distilled water only and then mounted on Parlodion-coated grids (Mallinckrodt Chemical Works, St. Louis, MO) in such a way that the remaining attached cell fragments were sandwiched between the replica and the copper grid.

Quantitation

The size of receptor clusters was determined by counting the number of gold particles associated in each distinguishable cluster on label fracture samples. Gold particles ≤ 20 nm apart were included in the same cluster. For each condition, in each experiment, an average of 1000 gold particles was counted on the first 20–25 fractured E-face of PMN encountered. The percentage of total gold particles in each size cluster was determined. The experiments were repeated on cells from three different individuals.

Acknowledgments

We are indebted to C. Maeder, G. Berthet, and A. Monod for skilled technical assistance. We thank Dr. J.-D. Vassalli and Dr. A. Perrelet for critical reading of the manuscript. This work has been supported by grants 31.26625.89 and 3.829.87 from the Swiss National Science Foundation and Dr O. Stendahl was supported by the Zyma Foundation for the advancement of medical and biological sciences.

Received: May 11, 1990.
Revised and accepted: October 23, 1990.

References

Abrahamson, D.R., and Fearon, D.T. (1983). Endocytosis of the C3b receptor of complement within coated pits in human polymorphonuclear leukocytes and monocytes. Lab. Invest. *48,* 162–168.

Andersson, T., Schlegel, W., Monod, A., Krause, K.-H., Stendahl, O., and Lew, D.P. (1986). Leukotriene B_4 stimulation of phagocytes results in the formation of inositol 1,4,5-triphosphate. Biochem. J., *240,* 333–340.

Barrowman, M.M., Cockcroft, S., Gomperts, B.D. (1987). Differential control of azurophil and specific granule exocytosis on Sendai-virus-permeabilized rabbit neutrophils. J. Physiol. *383,* 115–124.

Brandt, S.J., Dougherty, R.W., Lapetina, E.G., and Niedel, J.E. (1985). Pertussis toxin inhibits chemotactic peptide stimulated generation of inositol phosphates and lysosomal enzyme secretion in human leukemic (HL-60) cells. Proc. Natl. Acad. Sci. USA *82,* 3277–3280.

Brown, E.J. (1986). The role of extracellular matrix proteins in the control of phagocytosis. J. Leuk. Biol. *39,* 579–591.

Carpentier, J.-L., Gorden, P., Amherdt, M., Van Obberghen, E., Kahn, C.R., and Orci, L. (1978). ^{125}I-insulin binding to cultured human lymphocytes. Initial localization and fate of hormone determined by quantitative electron microscopic autoradiography. J. Clin. Invest. *61,* 1057–1070.

Carpentier, J.-L., Gorden, P., Freychet, P., Le Cam, A., and Orci, L. (1979). Lysosomal association of ^{125}I-insulin in isolated rat hepatocytes. Direct demonstration by quantitative electron microscopic autoradiography. J. Clin. Invest. *63,* 1249–1261.

Carpentier, J.-L., Van Obberghen, E., Gorden, P., and Orci, L. (1981). Surface redistribution of ^{125}I-insulin in cultured human lymphocytes. J. Cell Biol. *91,* 17–25.

Carpentier, J.-L., Van Obberghen, E., Gorden, P., and Orci, L. (1981). Binding, membrane redistribution, internalization and lysosomal association of ^{125}I-anti-insulin receptor anti-

body in IM-9 cultured human lymphocytes: a comparison with ^{125}I-insulin. Exp. Cell Res. *134,* 81–91.

Carpentier, J.-L., Gorden, P., Anderson, R.G.W., Goldstein, J.L., Brown, M.S., Cohen, S., and Orci, L. (1982). Co-localization of ^{125}I-Epidermal Growth Factor and ferritin low density lipoprotein in coated pits: a quantitative electron microscopic study in normal and mutant human fibroblasts. J. Cell Biol. *95,* 73–77.

Carpentier, J.-L., Brown, D., Iacopetta, B., and Orci, L. (1985). Detection of surface bound ligands by freeze-fracture autoradiography. J. Cell Biol. *101,* 887–890.

Carpentier, J.-L., Gazzano, H., Van Obberghen, E., Fehlmann, M., Freychet, P., and Orci, L. (1986). Intracellular pathway followed by the insulin receptor covalently coupled to ^{125}I-photoreactive insulin during internalization and recycling. J. Cell Biol. *102,* 989–996.

Carpentier, J.L., White, M.F., Orci, L., and Kahn, R.C. (1987). Direct visualization of the phosphorylated epidermal growth factor receptor during its internalization in A-431 cells. J. Cell Biol. *105,* 2751–2762.

Carpentier, J.L., Fehlmann, M., Van Obberghen, E., Gorden, P., and Orci, L. (1985). Redistribution of 125I-insulin on the surface of rat hepatocytes as a function of dissociation time. Diabetes *24,* 1002–1007.

Changelian, P.S., Jack, R.M., Collins, L.A., and Fearon, D.T. (1985). PMA induces the ligand-independent internalization of CR1 in human neutrophils. J. Immunol. *134,* 1851–1858.

Changelian, P.S., and Fearon, D.T. (1986). Tissue-specific phosphorylation of complement receptors CR1 and CR2. J. Exp. Med. *163,* 101–115.

Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A., and Hunter, T. (1984). C-kinase phosphorylates the epidermal growth factor receptors and reduces its epidermal growth-factor-stimulated tyrosine protein kinase activity. J. Biol. Chem. *259,* 2553–2558.

Detmers, P.A., Wright, S.D., Olsen, E., Kimball, B., and Cohn, Z.A. (1987). Aggregation of complement receptor on human neutrophils in the absence of ligand. J. Cell Biol. *105,* 1137–1145.

Di Virgilio, F., Lew, D.P., Pozzan, T. (1983). Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. Nature *310,* 691–693.

Di Virgilio, F., Vicentini, L.M., Treves, S., Riz, G., and Pozzan, T. (1985). Inositol phosphate formation in F.Met-Leu-Phe stimulated neutrophils does not require an increase in the cytosolic free calcium concentration. Biochem. J. *229,* 361–367.

Elsbach, P., and Weiss, J. (1988). Phagocytic cells: oxygen-independent antimicrobial systems. In: Inflammation Basic Principles and Clinical Correlates, eds. J.I. Gallin, I.M. Goldstein, R. Synderman. New York, Raven Press, 445–470.

Graham, R.C., and Karnovsky, M.J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. *14,* 291–301.

Helenius, A., Mellman, I., and Hubbard, A. (1983). Endosomes. Trends Biochem. Sci. *8,* 245–250.

Jack, R.M., Ezzell, R.M., Hartwig, J., and Fearon, D.T. (1986). Differential interaction of the C3b/C4b receptor and MHC class I with the cytoskeleton of human neutrophils. J. Immunol. *137,* 3996–4003.

Jacobs, S., Sahyoun, N.E., Saltiel, A.R., and Cuatrecasas, P. (1983). Phorbol esters stimulate the phosphorylation of receptors for insulin and somatomedin C. Proc. Natl. Acad. Sci. USA *80,* 6211–6213.

Korchak, H.M., Vienne, K., Rutherford, L.E., Winkenfeld, C., Finkelstein, M.C., and Weissmann, G. (1984). Stimulus response coupling in the human neutrophil II. Temporal analysis of changes in cytosolic calcium and calcium efflux. J. Biol. Chem. *259,* 4076–4082.

Lew, P.D., Wollheim, C.B., Waldvogel, F.A., and Pozzan, T. (1984). Modulation of cytosolic-free calcium transients by changes in intracellular calcium-buffering capacity: correlation with exocytosis and O_2^- production in human neutrophils. J. Cell Biol. *99,* 1212–1220.

Lew, P.D., Monod, A., Krause, K.-H., Waldvogel, F.A., Biden, T.J., and Schlegel, W. (1986a). The role of cytosolic calcium in the generation of inositol 1,4,5-triphosphate and inositol 1,3,4-triphosphate in HL-60 cells: differential effects of chemotactic peptide receptor stimulation at distinct Ca^{2+} levels. J. Biol. Chem. *261,* 13121–13127.

Lew, P.D., Monod, A., Waldvogel, F.A., Dewald, B., Baggiolini, M., and Pozzan, T. (1986b). Quantitative analysis of the cytosolic free calcium dependency of exocytosis from three subcellular compartments in intact neutrophils. J. Cell Biol. *102,* 2197–2204.

Lewis, W.H. (1931). Pinocytosis. Bull. Johns Hopkins Hosp. *49,* 17–27.

Malech, H.L., and Gallin, J.I. (1987). Neutrophils in human diseases. N. Engl. J. Med. *317,* 687–694.

May, W.S., Jacobs, S., and Cuatrecasas, P. (1984). Association of phorbol ester induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL60 cells. Proc. Natl. Acad. Sci. USA *81,* 2016–2020.

O'Shea, J.J., Brown, E.J., Gaither, T.A., Kakahashi, T., and Frank, M.M. (1985). Tumor-promoting esters induce rapid internalization of the C3b receptor via a cytoskeleton-dependent mechanism. J. Immunol. *135,* 1325–1330.

Paccaud, J.-P., Carpentier, J.-L., and Schifferli, K.-A. (1990). Difference in the clustering of complement receptor type one (CR1) on polymorphonuclear leucocytes and erythrocytes: effect on immune adherence. Eur. J. Immunol. *20,* 283–289.

Parham, P. (1986). Preparation and purification of active fragments from mouse monoclonal antibodies. In: Handbook of Experimental Immunology, ed. D.M. Weir. Oxford, UK: Blackwell Scientific Publications, 14.1.

Pauli, B.V., Weinstein, R.S., Some, L.W., and Alroy, J. (1977). Freeze-fracture of monolayer cultures. J. Cell. Biol. *72,* 763–769.

Pinto da Silva, P., and Kan, F.W.K. (1984). label fracture: a method for high reduction labeling of cell surfaces. J. Cell Biol. *99,* 1156–1161.

Pommier, C.G., O'Shea, J., Chused, T., Yancey, K., Frank, M.M., Takahaschi, T., and Brown, E.J. (1984). Studies of the fibronectin receptors of human peripheral blood leucocytes. J. Exp. Med. *159,* 137–151.

Pozzan, T., Lew, D.P., Wollheim, C.B., and Tsien, R.Y. (1983). Is cytosolic ionized calcium regulating neutrophil activation? Science *221,* 1413–1415.

Robinson, J.M., Badwey, J.A., Karnovsky, M.L., and Karnovsky, M.J. (1987). Cell surface dynamics of neutrophils stimulated with phorbol esters or retinoids. J. Cell Biol. *105,* 417–426.

Salisbury, J.L., and Keller, G.A. (1983). Structured investigations on the role of microfilaments in ligand translocation. Methods Enzymol. *98,* 368–375.

Sklar, A., Jesaitis, A.J., and Painter, R.G. (1984). The neutrophil N-formyl peptide receptor: dynamics of ligand-receptor interactions and their relationship to cellular responses. Contemp. Top. Immunobiol. *14,* 29–82.

Stossel, T.P. (1988). The mechanical responses of white blood cells. In: Inflammation basic principles and clinical correlates, eds. J.I. Gallin, I.M. Goldstein, R. Snyderman, New York: Raven Press, 325.

Synderman, R., Smith, C.D., and Verghese, M.W. (1986). Model for leukocyte regulation by chemoattractant receptors: roles of a guanine nucleotide regulatory protein and polyphosphoinositide metabolism. J. Leuk. Biol. *40,* 785–800.

Terris, S., Hofmann, C., and Steiner, D.F. (1979). Mode of uptake and degradation of ^{125}I-labeled insulin by isolated hepatocytes and H4 hepatoma cells. Can. J. Biochem. *57,* 457–468.

Tran, D., Carpentier, J.L., Sawano, F., Gorden, P., and Orci, L. (1987). Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. Proc. Natl. Acad. Sci. USA *84,* 7957–7961.

Turner, J.R., Tartakoff, A.M., and Berger, M. (1988). Intracellular degradation of the complement C3b/C4b receptor in the absence of ligand. J. Biol. Chem. *263,* 4914–4920.

Unkeless, J.C., and Wright, S.D. (1988). Phagocytic cells and complement receptors. In: Inflammation Basic Principles and Clinical Correlates, eds. J.I. Gallin, I.M. Goldstein, R. Snyderman, New York: Raven Press 343.

Weibel, E.R. (1969). Stereological principles for morphometry in electron microscope cytology. Int. Rev. Cytol. *26,* 235–302.

Willingham, T.C., and Yamada, S.S. (1978). A mechanism for the destruction of pinosomes in cultured fibroblasts piranhalysis. J. Cell Biol. *78,* 480–487.

Wills, E.J., Davies, P., Allison, A.C., and Haswell, A.D. (1972). Cytochalasin B fails to inhibit pinocytosis by macrophage. Nature *240,* 58–60.

Wright, S.D., and Silverstein, C. (1982). Tumor-promoting phorbol esters stimulate C3b and C3b′ receptor-mediated phagocytosis in cultured human monocytes. J. Exp. Med. *156,* 1149–1164.