Aggregation of Complement Receptors on Human Neutrophils in the Absence of Ligand

Patricia A. Detmers, Samuel D. Wright, Elizabeth Olsen, Beth Kimball, and Zanvil A. Cohn

Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021

Abstract. C3bi receptors (CR3) on human polymorphonuclear leukocytes (PMN) bind ligand-coated particles and promote their ingestion. The binding activity of CR3 is not constitutive but is transiently enabled by phorbol esters (Wright, S. D., and B. D. Meyer, 1986, *J. Immunol.* 136:1759–1764). Our observations indicate that the capacity of CR3 to bind ligand is tightly correlated with the degree of ligand-independent aggregation of the receptor in the plane of the membrane. Fixed PMN were labeled with anti-CR3 monoclonal antibodies and streptavidin colloidal gold before viewing in the electron microscope either en face or in thin section. On unstimulated PMN, gold particles marking CR3 were dispersed randomly. Stimulation of PMN for 25 min with phorbol myristate acetate (PMA) dramatically enhances binding of C3bi-coated particles, and the CR3 on such stimulated cells was observed in clusters containing more than six gold particles. CR3 was not aggregated over coated pits. After 50 min in PMA, the binding activity of CR3 falls, and the distribution of CR3 was again observed to be disperse. If a hydrophilic phorbol ester was washed away after a 20-min stimulation, binding activity remains elevated for at least 50 min, and CR3 remained aggregated. Thus, clustering of CR3 was temporally correlated with its ability to bind ligand and initiate phagocytosis. Unlike CR3, Fc receptors and HLA did not exhibit changes in their aggregation state in response to PMA. Treating PMN with formyl-methionyl-leucyl-phenylalanine, which enhances expression of CR3 but not its function, did not lead to aggregation of CR3. These observations suggest that a clustered configuration is a precondition necessary for binding ligand and signaling phagocytosis.

TRANSFER of information occurs bi-directionally across the plasma membrane. Ligation of receptors on the cell surface can influence a variety of intracellular processes, such as DNA synthesis, glucose metabolism, and reorganization of the cytoskeleton. Physiological changes within the cell may also alter the binding properties of receptors. For example, treatment of cells with phorbol esters has been reported to regulate the avidity of receptors for C3 (Wright and Meyer, 1986), the insulin receptor (Thomopoulos et al., 1982; Grunberger and Gordon, 1982), the epidermal growth factor receptor (King and Cuatrecasas, 1982), and α- and β-adrenergic receptors (Leeb-Lundberg et al., 1985; Kelleher et al., 1984).

The mechanisms by which receptors transmit signals into the cell are not fully understood, but, in several instances, ligands that initiate signals are also observed to cause aggregation of their receptors (King and Cuatrecasas, 1981). Moreover, signaling can be initiated by aggregating receptors for epidermal growth factor, IgE, or insulin with anti-receptor antibodies (Schreiber et al., 1983; Isersky et al., 1978; Jacobs et al., 1978). Monomeric IgE (Segal et al., 1977), CNBr fragments of epidermal growth factor (Schechter et al., 1979), and D-pyroGlu-D-Phe-D-Trp-D-Lys-GnRH, an antagonist of gonadotropin-releasing hormone (Conn et al., 1982), each bind to their respective receptors but fail to induce signaling. Cross-linking these ligands, however, enables them to initiate intracellular signals. Thus the view has emerged that receptor aggregation is necessary and sufficient for the transmission of signals to the cytoplasm (Kagey-Sobotka et al., 1982; Schlessinger, 1986; Kahn et al., 1978; Conn et al., 1985). We have investigated the reciprocal possibility that alterations within the cell may affect the activity of receptors for complement on the cell surface by changing their state of aggregation.

The C3bi receptor (CR3)[1] on human polymorphonuclear leukocytes (PMN) recognizes C3bi, a cleavage product of C3, bound to the surfaces of particles and initiates phagocytosis of the opsonized particles. CR3 is composed of two polypeptide chains, an α of 185 kD and a β of 95 kD as an $\alpha_1\beta_1$ dimer (Wright et al., 1983; Sanchez-Madrid et al., 1983). Recent work indicates that CR3 recognizes a region of C3bi that contains the triplet, Arg-Gly-Asp (RGD), and exhibits both structural and functional homology with the fibronectin receptor and related adhesion-promoting recep-

1. *Abbreviations used in this paper*: CR3, C3bi receptors; EC3bi, C3bi-coated erythrocytes; FMLP, formyl-methionyl-leucyl-phenylalanine; HLA, human leukocyte antigen; HSA, human serum albumin; NBCS, newborn calf serum; PD, PBS without $CaCl_2$ or $MgCl_2$; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes.

tors that recognize RGD (Wright et al., 1987; Law et al., 1987; Kishimoto et al., 1987).

The function of CR3 on monocytes and PMN can be regulated by several different stimuli (Wright and Griffin, 1985). For example, on PMN the activity of CR3 is enhanced by phorbol esters (Wright and Meyer, 1986). Resting cells express receptors on their cell surface that bind C3bi-coated erythrocytes (EC3bi) very poorly. Treatment of PMN with phorbol myristate acetate (PMA) increases binding of EC3bi sixfold, but the effect of PMA is transient, and binding of EC3bi falls to control levels after 50-min treatment with the phorbol ester. Phagocytosis of EC3bi is also regulated by PMA and exhibits an enhancement and depression congruent with binding. The rise and decline in binding and phagocytosis cannot be explained on the basis of changes in the expression of CR3 on the cell surface, since the number of receptors increases only twofold after 15 min in PMA and does not decline with longer incubation times. Thus the mechanism by which PMA alters the binding and signaling capacity of CR3 appears to involve primarily changes in the nature of the receptor, not in the number of receptors.

We have used a direct method, immunoelectron microscopy, to determine the distribution of CR3 before and during PMA treatment. Our observations suggest that enhanced activity of CR3 is closely correlated with the clustering of receptors in the plane of the membrane. Disaggregation of the receptors correlates with loss of receptor activity.

Materials and Methods

Preparation of PMN Leukocytes

Fresh blood was collected from healthy human donors, and PMN were isolated on Ficoll-Hypaque gradients (English and Anderson, 1974).

Reagents and Buffers

PMA, phorbol dibutyrate, formyl-methionyl-leucyl-phenylalanine (FMLP), Aprotinin, and ethyldimethylaminopropyl carbodiimide were supplied by Sigma Chemical Co., St. Louis, MO. Human serum albumin (HSA) was supplied by Worthington Biochemicals, Malvern, PA. Newborn calf serum (NBCS) was purchased from Gibco, Grand Island, NY. Glutaraldehyde and OsO_4 were purchased from Electron Microscopy Sciences, Fort Washington, PA. Buffers used were: PBS (137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 8 mM phosphate, pH 7.4) (Dulbecco and Vogt, 1954); PBS without $CaCl_2$ or $MgCl_2$ (PD); and PBS, 0.05% glucose, 0.5 mg/ml HSA, and 1:50 Aprotinin (HAP).

Antibodies

Monoclonal antibodies OKM1 and OKM10, directed against the α chain of CR3 (Wright et al., 1983), 3G8 directed against the Fc receptor ($FcR_{\gamma lo}$) (Fleit et al., 1982), and W6/32 directed against human leukocyte antigen (HLA) (Barnstable et al., 1978) were used in this study. Antibodies were purified on protein A-Sepharose columns and were biotinylated by standard procedures (Berman and Basch, 1980). Affinity-purified $F(ab)'_2$ anti-mouse immunoglobulin was supplied by Boehringer Mannheim Biochemicals, Indianapolis, IN, and was iodinated by the Iodogen procedure (Fraker and Speck, 1978) to specific activities of 10^6–10^7 cpm/μg.

Streptavidin–Colloidal Gold

For the initial phases of this study, streptavidin-conjugated colloidal gold was kindly provided by Dr. Frank Konings of Janssen Life Sciences Products. In addition, 6- and 10-nm colloidal gold was prepared by the method of Slot and Geuze (1985), and streptavidin was conjugated to it by standard procedures (DeMey, 1984). Each preparation of colloidal gold was characterized by electron microscopy to determine the size distribution of the particles and the degree of aggregate formation. Only those preparations with uniform size and having >99% of gold particles present as individuals were used for labeling experiments.

Binding of Antibody to Fixed Cells

To test for the ability of different fixation protocols to preserve antigenicity, we used a radioactive antibody-binding assay. PMN were plated in 60-well Terasaki plates and subjected to different test fixatives, including (*a*) dilute glutaraldehyde (0.05–0.3%) in PBS, 10 min at room temperature, (*b*) 1% ethyldimethylaminopropyl carbodiimide plus dilute glutaraldehyde (0.05–0.3%) in PBS/phosphate/Tris, pH 7.0 at room temperature for 7 min (Willingham, 1980), and (*c*) 20 mM ethylacetimidate, 3% paraformaldehyde, 2–10 min at room temperature, followed by 3% paraformaldehyde, 0.1% glutaraldehyde, 1 h on ice (Geiger et al., 1981). After two 15-min incubations in ice-cold 0.5 mg/ml $NaBH_4$ in PBS and 15 min in PD, 1% NBCS, the survival of antigens was measured as previously described (Wright and Meyer, 1986). Briefly, the fixed cells were exposed to monoclonal antibodies for 1 h, washed, exposed to ^{125}I-$F(ab)'_2$ anti-mouse IgG for 1 h, and washed again. After careful aspiration of the remaining wash solution, the individual wells were cut apart and counted in a gamma counter. Unfixed cells exposed to the same antibodies served as controls. We found that fixation in 1% ethyldimethylaminopropyl carbodiimide/0.2% glutaraldehyde (Willingham, 1980) was the most effective in preserving the antigenicity of a variety of epitopes: >90% of antibody binding was retained after fixation for the antibodies used in this study.

We used a similar assay to determine the expression of antigens on the surfaces of unfixed PMN after the cells had been stimulated with 30 ng/ml PMA for different lengths of time.

Immunoelectron Microscopy

A modification of the method of Hopkins (1985) was used to prepare whole-mounts of cells for immunoelectron microscopy. Glass coverslips were precoated with HSA by incubating them with buffer containing the protein (HAP) at room temperature for 20 min. HAP was removed by washing in PBS with 0.05% glucose, and PMN at 2×10^6 cells/ml were plated on the precoated coverslips. After exposure to the experimental conditions described in Results, the cells were fixed in 1% ethyldimethylaminopropyl carbodiimide/0.2% glutaraldehyde (Willingham, 1980), incubated 15 min in ice-cold 0.5 mg/ml $NaBH_4$ in PBS, and then in PD, 1% NBCS for 15 min. Cells were exposed to saturating concentrations of biotinylated monoclonal antibodies for 1 h on ice, washed in PD-NBCS, exposed to 10-nm streptavidin-conjugated colloidal gold, diluted to an OD_{520} of 0.3–0.5, for 2 h on ice and washed again in PD, 1% NBCS. Samples were then fixed at room temperature for 45 min in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4, and postfixed on ice for 15 min in 1% OsO_4 in the same buffer. After dehydration in ethanol, the samples were critical-point-dried and rotary shadowed with Pt-C in either a freeze-fracture apparatus (Balzers) or a vacuum evaporator (Edwards High Vacuum, Inc., Grand Island, NY). Replicas were removed with dilute hydrofluoric acid and picked up on formvar- and carbon-coated grids. Samples were viewed on a JEOL 100 CX microscope at 80 kV, and the peripheral areas of cells were photographed. The label observed on the cell periphery was found to be representative of labeling over the entire cell surface, as no differences in labeling were observed between peripheral and perinuclear regions of membrane on thin-sectioned samples (data not shown).

For thin-sectioned samples, PMN at 2×10^6 cells/ml were plated in plastic dishes precoated with HSA and fixed and labeled as for the preparation of replicas, with the exception that 6-nm streptavidin-conjugated colloidal gold was used for labeling. The cells were then fixed on ice in two changes of 0.8% glutaraldehyde/0.6% OsO_4 in 0.1 M Na cacodylate, pH 7.4, for 30 min and postfixed in 1% OsO_4 in the same buffer for 15 min (Hirsch and Fedorko, 1968). After dehydration in ethanol, monolayers were released from the plastic surface by adding propylene oxide (Steinman and Cohn, 1972), collected in microfuge tubes, pelleted, and embedded in Epon for thin-sectioning. Sections were stained in uranyl acetate and lead citrate before viewing.

The specificity of labeling was determined in control experiments in which the binding of biotinylated antibody was inhibited by a 70-fold excess of unbiotinylated antibody. Binding of streptavidin–colloidal gold to PMN under these conditions was undetectable (data not shown). Binding of streptavidin gold to PMN was less than four particles per cell when the biotinylated antibody was omitted from the labeling protocol for whole mounts.

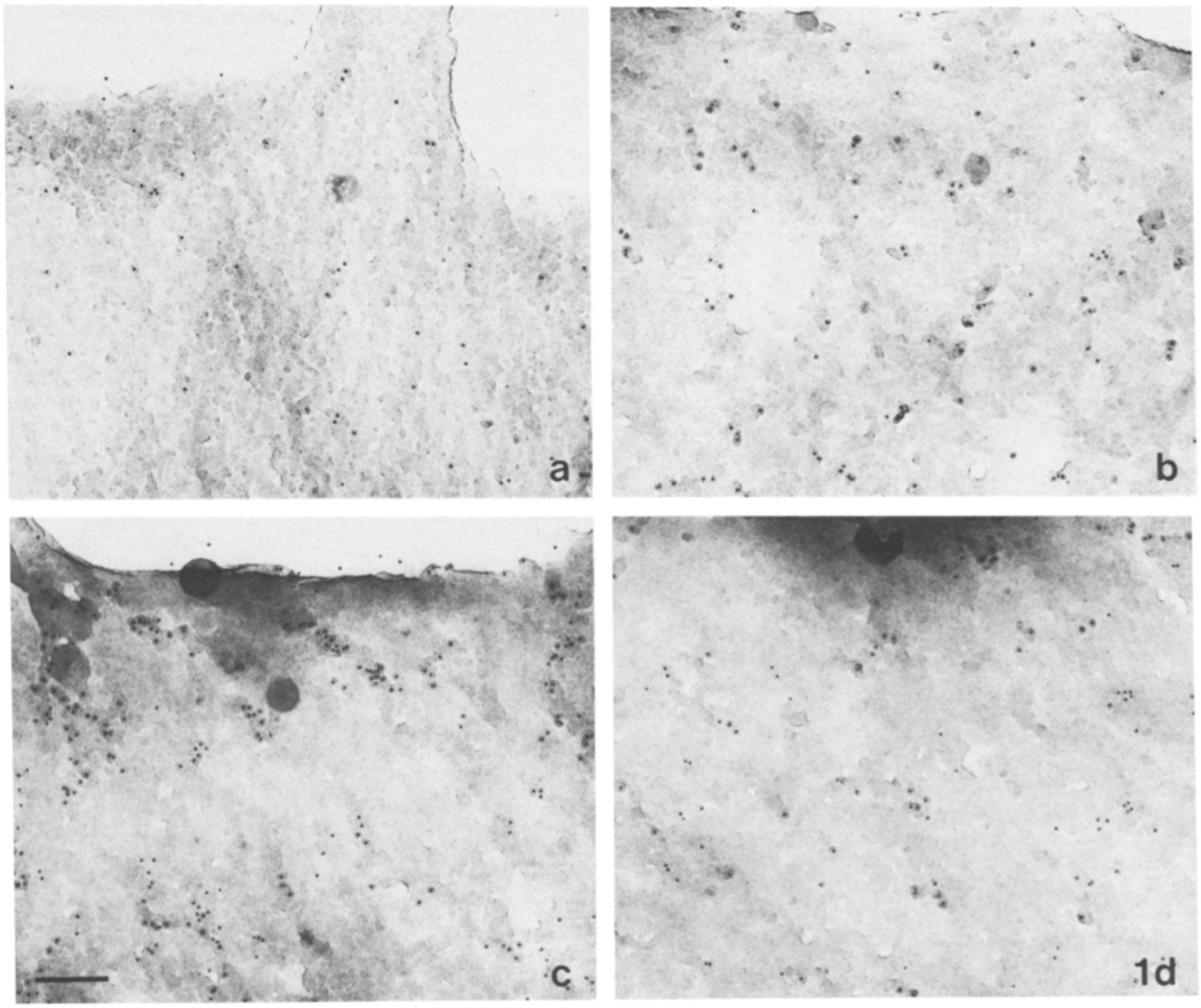


Figure 1. Immunolocalization of CR3 during treatment of PMN with PMA. PMN were incubated at 37°C for a total of 50 min, and at intervals 30 ng/ml PMA was added to the cells. Samples were then fixed and labeled as whole mounts with OKM10 and streptavidin gold as described in Materials and Methods. The length of exposure of cells to PMA was (*a*) 0 min (no PMA), (*b*) 10 min, (*c*) 25 min, and (*d*) 50 min. Bar, 0.2 μm.

Quantitation

The size of receptor clusters was determined by counting the number of gold particles associated in each distinguishable cluster on whole mount samples. To warrent inclusion in the same cluster, gold particles were no more than 30-nm apart. For each experimental condition an average of 4,000 gold particles was counted by an individual unaware of the experimental condition, and the percentage of total gold particles in each size of cluster was determined and graphed as a histogram. The data presented in this report are representative of at least three labeling experiments for each experimental condition.

Although the stoichiometry of binding of gold particles to receptors cannot be determined precisely, it is unlikely that individual receptors were labeled by more than one gold particle. Our experiments used monoclonal antibodies, so no more than one antibody should be bound per receptor. Each antibody molecule was biotinylated on at least one site, since the streptavidin gold adsorbed >90% of biotinylated OKM10 in control experiments (data not shown). The observation that labeling of CR3 on resting cells (e.g., Fig. 1 *a*) yielded single gold particles confirmed the assumption that antibodies bound no more than one gold particle. Thus the number of gold particles per cluster is proportional to the number of receptors per cluster, and clusters containing *n* gold particles contain at least *n* receptors.

The density of labeling was determined by counting the number of particles in each square (0.5 μm^2) of a grid laid over images of whole mount samples. At least seven areas from each of 30 cells were counted for each experimental condition. The density of labeling varied in different experiments and varied from cell to cell within an experiment. Thus, data from three separate experiments were averaged and the standard deviation determined.

Assays for Attachment and Phagocytosis of Ligand-coated Erythrocytes

Sheep erythrocytes (E) were coated with ~20,000 IgG/E or ~80,000 C3bi/E as previously described (Wright and Silverstein, 1982). The binding of these erythrocytes to monolayers of PMN was measured by adding 50 erythrocytes per PMN and incubating the preparation at 37°C for 30 min. Unbound erythrocytes were then washed away, and binding was scored by phase-contrast microscopy. The number of attached erythrocytes per 100 phagocytes is termed the Attachment Index (AI). Measurement of the Phagocytic Index (PI) was made in a similar fashion and was scored after lysing bound but uningested E by a brief exposure to distilled water (Wright and Silverstein, 1982). Control experiments indicated that uncoated erythrocytes were neither bound nor ingested by PMN (AI < 1, PI < 1).

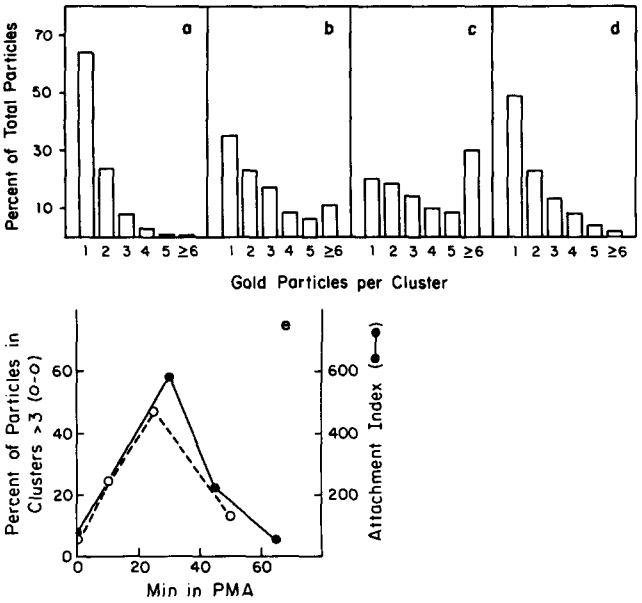


Figure 2. Quantitation of the size of CR3 clusters during treatment of PMN with PMA. PMN were treated and labeled as in Fig. 1. Quantitation of gold particles per cluster was performed as described in Materials and Methods. Cells were exposed to PMA for (*a*) 0 min (no PMA), (*b*) 10 min, (*c*) 25 min, and (*d*) 50 min. To compare the time course of receptor clustering with that of receptor activity, parallel cultures were prepared in Terasaki tissue culture wells. After the 50-min incubation period described above, C3bi-coated erythrocytes were added to all wells and incubation was continued for an additional 15 min. The attachment of the red cells to PMN was then scored as described in Materials and Methods (*solid circles* in *e*). Since binding of erythrocytes to PMN requires at least 15 min, rapid changes in receptor activity cannot be measured, and the amount of binding depicted at each time point represents binding events that occurred during the previous 15-min period. The percent of particles in clusters less than three was derived from the data in *a–d* (*open circles* in *e*).

Results

Distribution of CR3 on the Surface of Resting and Stimulated PMN

To observe the distribution of CR3, PMN were exposed to 30 ng/ml PMA for varying lengths of time, fixed and labeled as whole mounts with the monoclonal antibody OKM10 and streptavidin–colloidal gold as described in Materials and Methods. On resting cells CR3 was randomly distributed, with many of the gold particles present as individuals (Fig. 1 *a*). After stimulation of the cells for 10 min with PMA, CR3 appeared more aggregated (Fig. 1 *b*), and by 25 min large clusters of gold particles were apparent (Fig. 1 *c*). By this time receptors exhibit maximum capacity to bind ligand and promote phagocytosis (Wright and Meyer, 1986). After 50 min in PMA, the capacity of CR3 to bind ligand and signal phagocytosis declines to baseline levels (Wright and Meyer, 1986). At this time the gold particles were again present as individuals, indicating CR3 had disaggregated (Fig. 1 *d*). These results were confirmed in separate labeling experiments using OKM1, an antibody against a different epitope on CR3 (data not shown).

Quantitation of the gold particles per cluster for each time point confirmed the impression obtained from the electron micrographs. On resting cells 60% of the gold particles were present as individuals, and clusters of more than six gold particles were barely detectable (Fig. 2 *a*). Individual gold particles declined to 35% by 10 min in PMA, with a corresponding increase in the number of gold particles in clusters of three or more (Fig. 2 *b*). By 25 min in PMA there was a further decline in individual particles to 20%, while the percent of particles in clusters of six or more increased to 30% (Fig. 2 *c*). This trend was reversed at 50 min when 50% of the particles were once again present as individuals (Fig. 2 *d*). The time course of aggregation of CR3 correlated well with the increase in binding activity of this receptor, while disaggregation correlated with the loss of activity at longer incubations (Fig. 2 *e*).

Table I. Density of CR3 on the Surface of PMN during Treatment with PMA

Time in PMA	Density of label
min	*particles/μm²*
0 (no PMA)	16.3 ± 17.6
10	32.2 ± 18.6
25	37.9 ± 21.7
50	21.8 ± 13.8

PMN were treated with PMA and labeled with OKM10 as described in Fig. 1. Density of label was determined as described in Materials and Methods.

The density of gold labeling was measured on samples of PMN treated with PMA and labeled with OKM10 (Table I). After 10 min in PMA there was a twofold increase in the density of label on the cell surface. Receptor density remained unchanged at 25 min, and declined slightly after 50 min. Since these changes in receptor density were relatively small and were not temporally correlated with aggregation of CR3, it is unlikely that receptor aggregation was an artifact caused by changes in receptor density (see Discussion).

Phorbol dibutyrate (PDB) is a hydrophilic analogue of PMA that has similar transient effects on the binding and

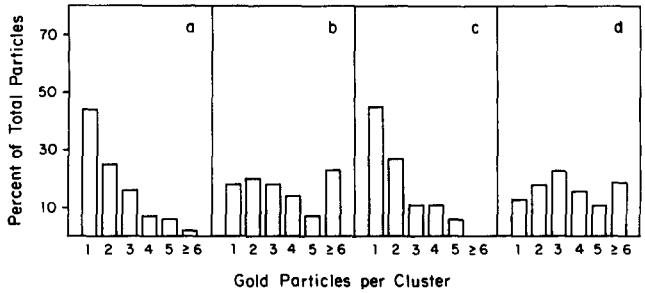


Figure 3. Quantitation of the size of CR3 clusters during treatment of PMN with PDB and after removal of the phorbol ester. PMN were incubated for a total of 50 min at 37°C with 300 ng/ml PDB added as follows: (*a*) no PDB, (*b*) PDB added for the last 25 min, (*c*) PDB added for 20 min, washed, PDB readded for another 30 min, (*d*) PDB added for 20 min, washed, buffer (HAP) added for the last 30 min. To effect complete removal of the PDB at the wash step described in *c* and *d*, cells were incubated for 15 min at 20°C in the presence of a large excess of protein-containing buffer (HAP). Samples were labeled as whole mounts with OKM1 and streptavidin gold, and quantitation of gold particles per cluster was performed as described in Materials and Methods.

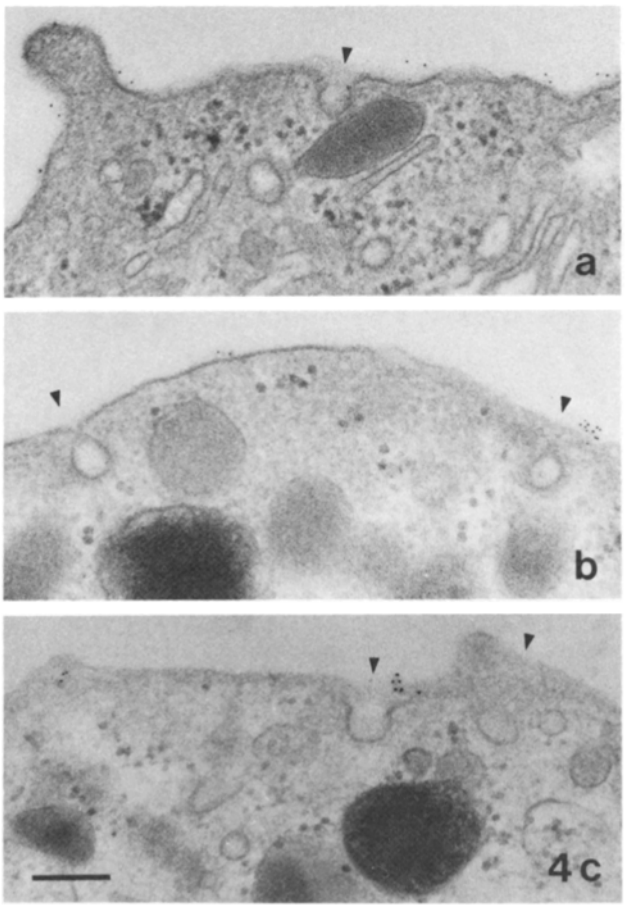


Figure 4. CR3 is not associated with coated pits. PMN were plated and incubated for 45 min at 37°C before labeling with OKM10 and preparation for thin sectioning as described in Materials and Methods. *a–c* show representative coated pits (*arrowheads*) from resting cells. Bar, 0.2 μm.

phagocytosis-promoting activities of CR3. The loss of receptor activity observed between 20 and 50 min of incubation requires the continuous presence of phorbol ester, because if the PDB is washed away from the cells after 20 min, the capacity of receptors to bind ligand remains at high levels (Wright and Meyer, 1986). To determine if the disaggregation of receptors between 25 to 50 min could be prevented by removing the phorbol ester, we incubated PMN for 20 min with 300 ng/ml PDB, then replaced the phorbol ester with buffer and incubated the cells for a further 30 min. Whole-mount samples were labeled with OKM1 and streptavidin gold as described in Materials and Methods. On PMN that were not exposed to PDB, CR3 was randomly distributed (Fig. 3 *a*). After stimulation with PDB for 25 min, gold particles marking the location of CR3 were clustered (Fig. 3 *b*), but stimulation of cells with PDB for the full 50 min caused subsequent disaggregation (Fig. 3 *c*). On samples from which the PDB was removed after 20 min and replaced with buffer for the succeeding 30-min incubation, CR3 remained clustered (Fig. 3 *d*), again demonstrating a correlation between the state of aggregation and the activity of the receptor.

CR3 Is Not Associated with Coated Pits

To determine if CR3 was present over coated pits in resting cells and whether the aggregates of CR3 observed in response to PMA were forming over coated pits, we labeled CR3 on the surfaces of PMN and prepared thin sections of the labeled cells. Label was observed randomly distributed over the apical surface of the cell but was not found within coated pits on resting cells (Fig. 4). Out of 34 coated pits observed on resting cells, none contained label for CR3. CR3 also did not cluster over coated pits during treatment with PMA: Only 1 out of 26 coated pits observed contained label after either 10 min or 25 min in 30 ng/ml PMA.

Distribution of Other Surface Antigens on PMN

To determine whether the aggregation of CR3 in response to phorbol esters was specific for that antigen or a general phenomenon of PMN surface proteins, we observed the distribution of HLA-A,B,C (MHC Class I) and Fc receptor ($FcR_{\gamma lo}$) on PMN surfaces during incubations with PMA. The expression of HLA on the surfaces of PMN did not change in response to treatment with PMA (data not shown). Whole mount samples labeled with W6/32 showed HLA to be present in a disaggregated state, homogeneously distributed over the cell surface at all times of incubation with PMA (Fig. 5).

$FcR_{\gamma lo}$ on resting PMN constitutively promotes binding and phagocytosis of IgG-coated particles, but the activity of $FcR_{\gamma lo}$ declines in the presence of PMA (Wright and Meyer, 1986). Labeling of $FcR_{\gamma lo}$ on resting cells was very dense, and, in contrast with HLA, the receptor exhibited a clustered distribution (Fig. 6 *a*). The amount of $FcR_{\gamma lo}$ on the surface of PMN decreases several-fold during treatment with PMA (Wright and Meyer, 1986), and we observed a decline in the density of labeling with increasing time of incubation in PMA that was consistent with this observation (Fig. 6 *b*). Essentially no label above background was detectable on cells after 50 min in PMA. Small clusters of gold particles remained present as long as labeling could be observed, suggesting that $FcR_{\gamma lo}$ may be present in a constitutive state of aggregation.

Distribution of CR3 on PMN Treated with FMLP

The chemotactic peptide FMLP causes an increase in the expression of CR3 on the surface of PMN (Berger et al., 1984;

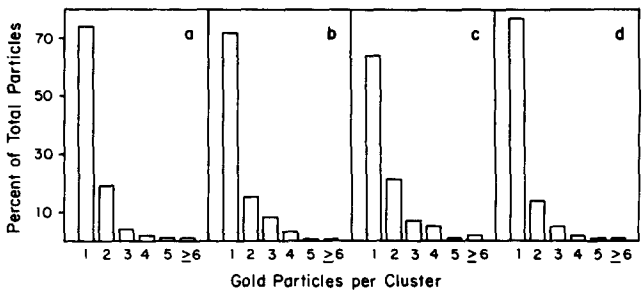


Figure 5. Distribution of HLA-A,B,C molecules on the surface of PMN. PMN were incubated at 37°C for a total of 50 min with 30 ng/ml PMA added at intervals during the incubation. Samples were labeled as whole mounts with W6/32 and streptavidin gold as described in Materials and Methods. Cells were exposed to PMA for (*a*) 0 min (no PMA), (*b*) 10 min, (*c*) 25 min, and (*d*) 50 min.

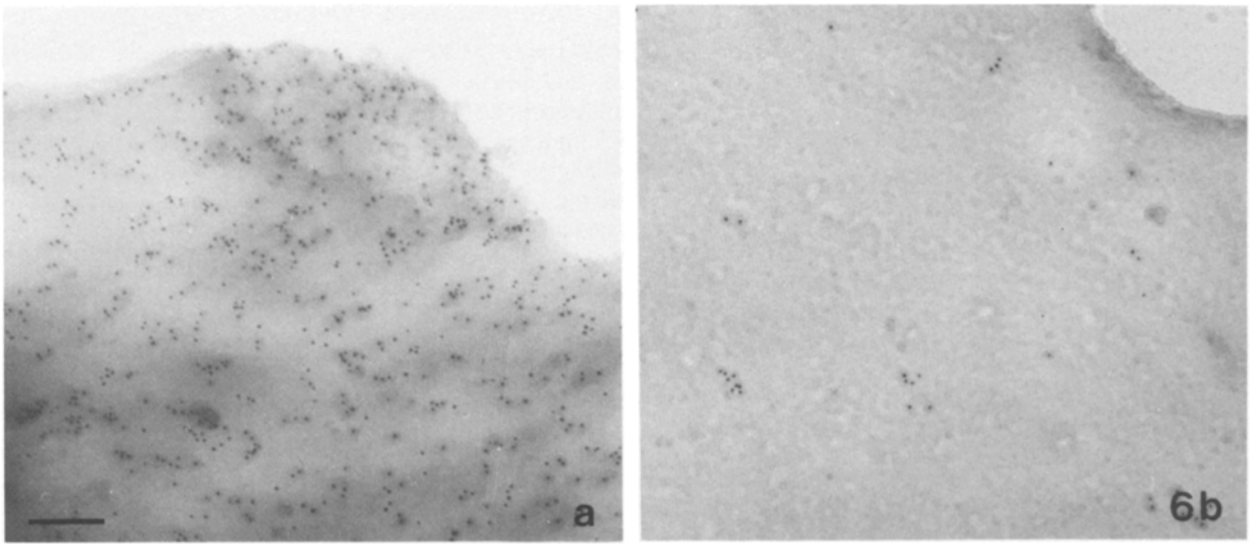


Figure 6. Immunolocalization of FcR_{ylo}. PMN were incubated at 37°C for 50 min. The final 25 min of the incubation were done in the absence (*a*) or the presence (*b*) of 30 ng/ml PMA. Samples were labeled as whole mounts with 3G8 and streptavidin gold as described in Materials and Methods. Bar, 0.2 μm.

O'Shea et al., 1985). We have observed, however, that stimulation with FMLP did not enhance the capacity of PMN to bind or ingest EC3bi (Table II), although phagocytosis of EIgG was enhanced about twofold. Thus, FMLP alters the number of CR3 on the cell surface without enhancing its activity. To observe whether FMLP caused aggregation of CR3, PMN were treated with 10^{-7} M FMLP for 10 or 20 min before labeling with OKM10 and streptavidin gold. Cells treated with FMLP exhibited a twofold increase in the density of label present on the cell surface (compare Fig. 7, *a* and *b*, and Table III). However, FMLP did not cause aggregation of CR3 (Fig. 7 and 8). Thus aggregation is not a consequence of receptor externalization, but appears tightly correlated with enhanced receptor activity.

FMLP causes PMN to polarize, and the receptors for FMLP have been observed to be preferentially distributed on the forward half of polarized cells (Sullivan et al., 1984). We observed strong polarization of cells in response to FMLP, but CR3 appeared to remain evenly distributed (Fig. 7, *b* and *c*). In blind comparisons, no difference in the pattern of labeling could be observed over different parts of the cell. The same results were obtained with 5×10^{-7} M FMLP (data not shown). Counts of the density of labeling over different areas of polarized cells revealed a slightly higher density of CR3 in the uropod region (Table III).

Table II. Attachment and Phagocytosis of Ligand-coated Erythrocytes by PMN Treated with FMLP or PMA

	Attachment index	Phagocytic index	
Stimulus	EC3bi	EC3bi	EIgG
–	127	9	185
FMLP	115	22	376
PMA	485	229	161

PMN were allowed to spread for 30 min at 37°C. The indicated ligand-coated erythrocytes were then added simultaneously with FMLP (5×10^{-7} M) or PMA (30 ng/ml), and, after 45 min at 37°C, the attachment and phagocytosis of erythrocytes was measured as described in Materials and Methods. Results shown are representative of three separate experiments.

Discussion

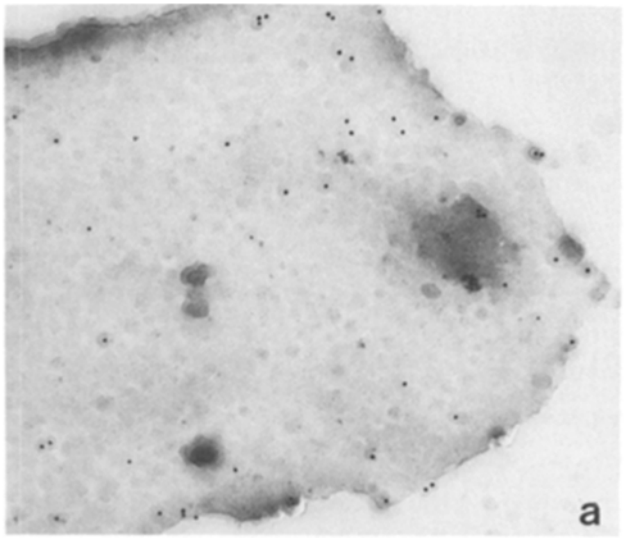
We have investigated the mechanism by which the binding capacity of CR3 is regulated. Our observations indicate that the capacity of CR3 to efficiently bind ligand is tightly correlated with the state of aggregation of the receptor in the plane of the membrane. Resting cells have randomly distributed receptors and very low capacity to bind C3bi-coated particles. Upon stimulation of the cells with PMA, the capacity to bind C3bi rises with a time course coincident with aggregation of the receptor. Upon further incubation with phorbol ester, the binding activity of CR3 declines with a time course coincident with disaggregation of receptors.

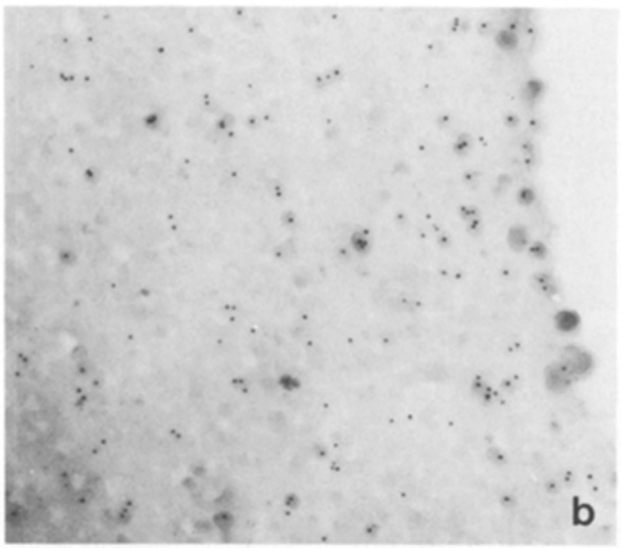
Stimulation of PMN with PMA causes an increase in the expression of CR3 on the cell surface, presumably due to the release of specific granules, which appear to comprise an intracellular store of CR3 (Todd et al., 1984; O'Shea et al., 1985). We do not know the state of aggregation of CR3 within the specific granules before externalization, and the possibility exists that receptors are deployed in a pre-aggregated state in response to PMA. Our observations suggest, however, that this is most likely not the case: After 10 min in PMA, both the total amount of cell surface CR3 (Wright and Meyer, 1986) and the density of CR3 (Table I) have increased, but additional time was required before maximal aggregation of CR3 was observed (Fig. 2). This suggests that the receptors

Table III. Density of CR3 on the Surface of PMN during Treatment with FMLP

Treatment	Area of cell	Density of label
		particles/μm²
No FMLP	Periphery	17.5 ± 11.0
10 min FMLP	Leading edge	33.3 ± 21.0
	Uropod	46.3 ± 28.0
	Other areas of periphery	34.3 ± 25.0

PMN were treated with FMLP and labeled with OKM10 as described in Fig. 7. Density of label was determined as described in Materials and Methods.





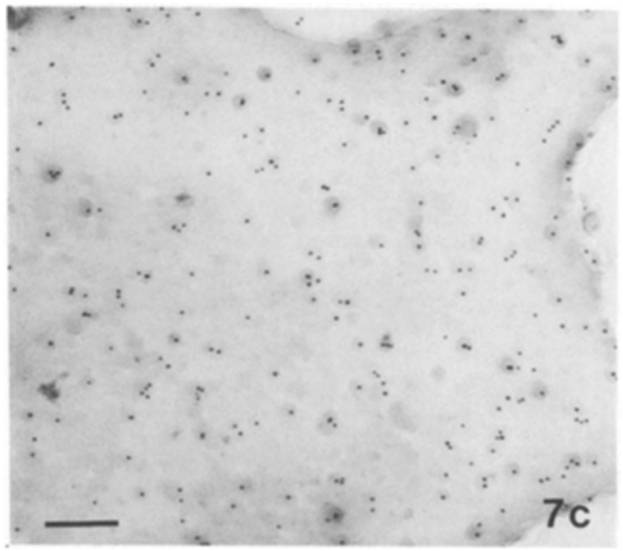


Figure 7. Immunolocalization of CR3 on cells treated with FMLP. PMN were incubated at 37°C for a total of 50 min. The final 10 min of the incubation were done in the absence (*a*) or presence (*b* and *c*) of 10^{-7} M FMLP. Samples were labeled as whole mounts with OKM10 and streptavidin gold as described in Materials and Methods. Areas from either the leading edge (*b*) or uropod region (*c*) of a well-polarized cell are shown. Bar, 0.2 μm.

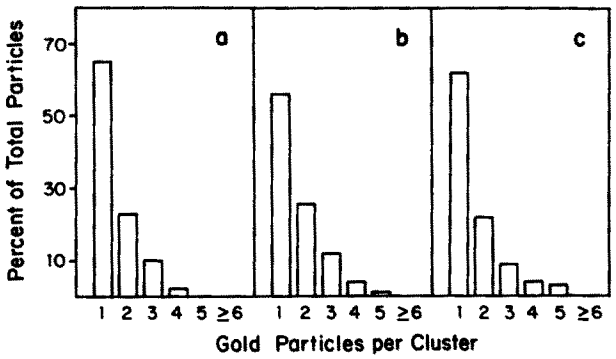


Figure 8. Quantitation of the size of CR3 clusters during treatment of PMN with FMLP. PMN were treated and labeled as in Fig. 7. Quantitation of gold particles per cluster was performed as described in Materials and Methods. Cells were exposed to FMLP for (*a*) 0 min (no FMLP), (*b*) 10 min, or (*c*) 20 min.

are deployed in a non-aggregated state, and that they then form clusters once they are on the cell surface. The aggregation of CR3 is also not an automatic consequence of externalization of receptors or of changes in receptor density, since stimulation of cells with FMLP caused externalization of intracellular receptors and raised receptor density (Table III) but did not lead to aggregation (Fig. 8) or enhanced receptor activity (Table II).

The mechanism underlying aggregation is not clear, but several possibilities can be ruled out. Aggregation is not a consequence of interaction with extracellular ligand, since aggregation was observed before the addition of ligand. Aggregation is probably not a general property of cell membrane proteins of PMN, since a control transmembrane protein, HLA, does not redistribute under the conditions that lead to aggregation of CR3. Since the gathering of receptors into a nonrandom configuration is likely to be a thermodynamically unfavorable reaction, we presume that aggregation is an energy-dependent process. PMA is known to activate protein kinase C (Castagna et al., 1982; Niedel et al., 1983), and we speculate that a phosphorylation event is involved in controlling the aggregation of CR3.

Receptors for low density lipoprotein are preclustered into coated pits in the absence of ligand (Anderson et al., 1976, 1977), and epidermal growth factor receptors aggregate over coated pits after binding of ligand (Haigler et al., 1979), but aggregation of CR3 does not appear to involve clathrin. The clustering of CR3 did not occur over coated pits, and CR3 on resting PMN was not present in coated pits. Alternative intracellular proteins that may interact with CR3 are the elements of the actin-based cytoskeleton. CR3 is structurally homologous with the fibronectin receptor (Wright et al., 1987; Law et al., 1987; Kishimoto et al., 1987), a molecule known to interact with the actin-based cytoskeleton when aggregated in "adhesion plaques" (Hynes and Destree, 1978; Ali and Hynes, 1977). The possibility that CR3 is similarly associated with the cytoskeleton is currently under investigation.

How might aggregation of CR3 control its interaction with extracellular ligand? The interaction of CR3 with mono-

meric ligand is apparently of very low affinity, since soluble C3bi is not bound by CR3 and must be attached to a surface to be recognized by the receptor. Moreover, the distribution of C3bi on a surface contributes to the efficiency of recognition; particles coated with C3bi in a random distribution are bound poorly by phagocytes but particles on which the C3bi is clustered are very efficiently bound (Hermanowski-Vosatka, A., P. A. Detmers, O. Goetze, S. C. Silverstein and S. D. Wright, manuscript in preparation). Therefore the simplest explanation for the role of clustering of receptors and ligand is to enhance the avidity of their interaction by lowering the apparent off rate. Disassociation of a cluster of ligand from a cluster of receptors will only occur after simultaneous disassociation of all the individual receptor–ligand pairs. Experimental measurements of the disassociation of monovalent and divalent antibodies from cells indicate that divalent antibodies disassociate at least 10-fold more slowly than monovalent ones (Perelson and DeLisi, 1980; Mason and Williams, 1980). Associations between surface-bound ligand and clusters of more than six receptors, such as we have observed, should therefore exhibit enormously enhanced stability. This interpretation does not exclude the possibility that conformational changes that influence binding could accompany aggregation of CR3.

The labeling patterns for FcR_{ylo} and CR3 were markedly different on both resting and stimulated PMN, thus indicating that anti-CR3 was not binding via its Fc domain. Resting PMN are capable of binding and phagocytosing EIgG very efficiently (Wright and Meyer, 1986), and we observed that FcR_{ylo} was present in a clustered configuration on such cells. Like CR3, FcR_{ylo} binds monomeric ligand with low affinity, and only aggregates of IgG are effectively recognized by PMN (Kurlander and Batker, 1982). This difference in affinity is potentially explained by the clustered distribution of FcR_{ylo}, and this may represent another example in which the aggregation state of ligand and receptor controls their interactions.

Since it is likely that PMA acts on an intracellular target, protein kinase C, our results suggest that aggregation of receptors may be a mechanism by which intracellular changes are communicated to the cell surface. The reciprocal may also occur: Aggregation of other receptors after addition of ligand or anti-receptor antibodies may create high affinity sites on the cytoplasmic side of the plasma membrane for the binding of regulatory proteins. These studies may therefore provide a model for understanding how ligation of receptors at the cell surface and the consequent aggregation of the receptors may signal intracellular events.

We wish to express our appreciation to Dr. Frank Konings and Janssen Life Sciences Products for the gift of streptavidin-conjugated colloidal gold. We also thank Dr. David Phillips of the Population Council for kindly letting us use his Edwards vacuum evaporator, Ms. Michelle Jong for technical assistance, Ms. Judy Adams for photographic assistance, and Drs. Ralph Steinman and Alan Aderem for suggestions during the preparation of this manuscript.

This work was supported by a Grant-in-Aid from the American Heart Association and with funds contributed in part by the American Heart Association, Florida Affiliate (P. Detmers), and by grants AI2203 (S. Wright) and AI07012 (Z. Cohn) from the U. S. Public Health Service and JFRA-103 from the American Cancer Society (S. Wright). P. Detmers is an Arthritis Foundation Investigator.

Received for publication 5 February 1987, and in revised form 14 May 1987.

References

Ali, I. U., and R. O. Hynes. 1977. Effects of cytochalasin B and colchicine on attachment of a major surface protein of fibroblasts. *Biochim. Biophys. Acta.* 471:16–24.

Anderson, R. G. W., M. S. Brown, and J. L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell.* 10:351–364.

Anderson, R. G. W., J. L. Goldstein, and M. S. Brown. 1976. Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. *Proc. Natl. Acad. Sci. USA.* 73:2434–2438.

Barnstable, C. J.,W.F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Zeigler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens: new tools for genetic analysis. *Cell.* 14:9–20.

Berger, M., J. O'Shea, A. S. Cross, T. M. Folks, T. M. Chused, E. J. Brown, and M. M. Frank. 1984. Human neutrophils increase expression of C3bi as well as C3b receptors upon activation. *J. Clin. Invest.* 74:1566–1571.

Berman, J. W., and R. S. Basch. 1980. Amplification of the biotin-avidin immunofluorescence technique. *J. Immunol. Methods.* 36:335–338.

Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847–7851.

Conn, P. M., D. C. Rogers, S. G. Seay, H. Jinnah, M. Bates, and D. Luscher. 1985. Regulation of gonadotropin release, GnRH receptors, and gonadotrope responsiveness: a role for GnRH receptor microaggregation. *J. Cell. Biochem.* 27:13–21.

Conn, P. M., D. C. Rogers, J. M. Stewart, J. Niedel, and T. Sheffield. 1982. Conversion of a gonadotropin-releasing hormone antagonist to an agonist. *Nature (Lond.).* 296:653–655.

DeMey, J. 1984. Colloidal gold as marker and tracer in light and electron microscopy. *EMSA Bull.* 14:54–66.

Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* 99:167–182.

English, D., and B. R. Anderson. 1974. Single-step separation of red blood cells, granulocytes, and mononuclear phagocytes on discontinuous density gradients of Ficoll-Hypaque. *J. Immunol. Methods.* 5:249–252.

Fleit, H. B., S. D. Wright, and J. C. Unkeless. 1982. Human neutrophil Fc_γ receptor distribution and structure. *Proc. Natl. Acad. Sci. USA.* 79:3275–3279.

Fraker, P. J., and J. C. Speck. 1978. Protein and cell membrane iodination with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849–857.

Geiger, B., A. H. Dutton, K.T. Tokuyasu, and S. J. Singer. 1981. Immunoelectron microscope studies of membrane-microfilament interactions: Distributions of alpha-actinin, tropomyosin, and vinculin in intestinal epithelial brush border and chicken gizzard smooth muscle cells. *J. Cell Biol.* 91:614–628.

Grunberger, G., and P. Gordon. 1982. Affinity alteration of insulin receptor induced by a phorbol ester. *Am. J. Physiol.* 243:E319–E324.

Haigler, H. T., J. A. McKanna, and S. Cohen. 1979. Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J. Cell Biol.* 81:382–395.

Hirsch, J. G., and M. E. Fedorko. 1968. Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "postfixation" in uranyl acetate. *J. Cell Biol.* 38:615–627.

Hopkins, C. R. 1985. The appearance and internalization of transferrin receptors at the margins of spreading human tumor cells. *Cell.* 40:199–208.

Hynes, R. O., and A. T. Destree. 1978. Relationship between fibronectin (LETS protein) and actin. *Cell.* 15:875–886.

Isersky, C., J. D. Taurog, G. Poy, and H. Metzger. 1978. Triggering of cultured neoplastic mast cells by antibodies to the receptor for IgE. *J. Immunol.* 121:549–558.

Jacobs, S., K. J. Chang, and P. Cuatrecasas. 1978. Antibodies to purified insulin receptor have insulin-like activity. *Science (Wash. DC).* 200:1283–1284.

Kagey-Sobotka, A., D. W. MacGlashan, and L. M. Lichtenstein. 1982. Role of receptor aggregation in triggering IgE-mediated reactions. *Fed. Proc.* 41: 12–16.

Kahn, C. R., K. L. Baird, D. B. Jarrett, and J. S. Flier. 1978. Direct demonstration that receptor crosslinking or aggregation is important in insulin action. *Proc. Natl. Acad. Sci. USA.* 75:4209–4213.

Kelleher, D. J., J. E. Pessin, A. E. Ruoho, and G. L. Johnson. 1984. Phorbol ester induces desensitization of adenylate cyclase and phosphorylation of the beta-adrenergic receptor in turkey erythrocytes. *Proc. Natl. Acad. Sci. USA.* 81:4316–4320.

King, A. C., and P. Cuatrecasas. 1981. Peptide hormone-induced receptor mobility, aggregation, and internalization. *N. Eng. J. Med.* 305:77–88.

King, A. C., and P. Cuatrecasas. 1982. Resolution of high and low affinity epidermal growth factor receptors. *J. Biol. Chem.* 257:3053–3060.

Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1987. Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell.* 48:681–690.

Kurlander, R. J., and J. Batker. 1982. The binding of human immunoglobulin G1 monomer and small, covalently cross-linked polymers of immunoglobu-

lin G1 to human peripheral blood monocytes and polymorphonuclear leukocytes. *J. Clin. Invest.* 69:1–8.

Law, S. K. A., J. Gagnon, J. E. K. Hildreth, C. E. Wells, A. C. Willis, and A. J. Wong. 1987. The primary structure of the β-subunit of the cell surface adhesion glycoproteins LFA-1, CR3 and p150,95 and its relationship to the fibronectin receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:915–919.

Leeb-Lundberg, L. M. F., S. Cotecchia, J. W. Lomasney, J. F. DeBernardis, R. J. Lefkowitz, and M. G. Caron. 1985. Phorbol esters promote alpha1-adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc. Natl. Acad. Sci. USA.* 82:5651–5655.

Mason, D. W., and A. F. Williams. 1980. The kinetics of antibody binding to membrane antigens in solution and at the cell surface. *Biochem. J.* 187:1–20.

Niedel, J. E., L. J. Kuhn, and G. R. Vandenbark. 1983. Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA.* 80:36–40.

O'Shea, J., E. J. Brown, B. E. Seligmann, J. A. Metcalf, M. M. Frank, and J. I. Gallin. 1985. Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. *J. Immunol.* 134:2580–2587.

Perelson, A. S., and C. DeLisi. 1980. Receptor clustering on a cell surface. I. Theory of receptor cross-linking by ligands bearing two chemically identical functional groups. *Math. Biosci.* 48:71–110.

Sanchez-Madrid, F., J. A. Nagy, E. Robbins, P. Simon, and T. A. Springer. 1983. Characterization of a human leukocyte differentiation antigen family with distinct α subunits and a common β subunit: the lymphocyte-function associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac1), and the p150,95 molecule. *J. Exp. Med.* 158:1785–1803.

Schechter, Y., L. Hernaez, J. Schlessinger, and P. Cuatrecasas. 1979. Local aggregation of hormone-receptor complexes is required for activation by epidermal growth factor. *Nature (Lond.).* 278:835–838.

Schlessinger, J. 1986. Allosteric regulation of the epidermal growth factor receptor kinase. *J. Cell Biol.* 103:2067–2072.

Schreiber, A. B., T. A. Libermann, I. Lax, Y. Yarden, and J. Schlessinger. 1983. Biological role of epidermal growth factor-receptor clustering. Investigation with monoclonal anti-receptor antibodies. *J. Biol. Chem.* 258:846–853.

Segal, D. M., J. D. Taurog, and H. Metzger. 1977. Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. *Proc. Natl. Acad. Sci. USA.* 74:2993–2997.

Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multiple-labelling cytochemistry. *Eur. J. Cell Biol.* 38:87–93.

Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. *J. Cell Biol.* 55: 186–204.

Sullivan, S. J., G. Daukas, and S. H. Zigmond. 1984. Asymmetric distribution of the chemotactic peptide receptor on polymorphonuclear leukocytes. *J. Cell Biol.* 99:1461–1467.

Thomopoulos, P., U. Testa, M. F. Gourdin, C. Hervy, M. Titeux, and W. Vainchenker. 1982. Inhibition of insulin receptor binding by phorbol esters. *Eur. J. Biochem.* 129:389–393.

Todd, R. F., III, M. A. Arnaout, R. E. Rosin, C. A. Crowley, W. A. Peters, and B. M. Babior. 1984. Subcellular localization of the large subunit of Mol (Mol': formerly gp 110), a surface glycoprotein associated with neutrophil adhesion. *J. Clin. Invest.* 74:1280–1290.

Willingham, M. C. 1980. Electron microscopic immunocytochemical localization of intracellular antigens in cultured cells: the EGS and ferritin bridge procedures. *Histochem. J.* 12:419–434.

Wright, S. D., and F. M. Griffin, Jr. 1985. Activation of phagocytic cells' C3 receptors for phagocytosis. *J. Leuk. Biol.* 38:327–339.

Wright, S. D., and B. C. Meyer. 1986. Phorbol esters cause sequential activation and deactivation of complement receptors on polymorphonuclear leukocytes. *J. Immunol.* 136:1759–1764.

Wright, S. D., P. E. Rao, W. C. Van Voorhis, L. S. Craigmyle, K. Iida, M. A. Talle, G. Goldstein, and S. C. Silverstein. 1983. Identification of the C3bi receptor on human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:5699–5703.

Wright, S. D., P. A. Reddy, M. T. C. Jong, and B. W. Erickson. 1987. The C3bi receptor (CR3) recognizes a region of complement protein C3 containing the sequence Arg-Gly-Asp. *Proc. Natl. Acad. Sci. USA.* 84:1965–1968.

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