Intracellular Degradation of the Complement C3b/C4b Receptor in the Absence of Ligand*

(Received for publication, September 8, 1987)

Jerroid R. Turner‡, Alan M. Tartakoff, and Melvin Berger§
From the Departments of Pathology and Pediatrics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Human neutrophils (PMN) respond to various soluble stimuli by translocating intracellular complement C3b/C4b receptors (CR1) to the cell surface. Ligand-independent internalization of surface CR1 has been demonstrated previously, but the fate of total cellular CR1 during PMN stimulation has not been determined. In order to study the fate of CR1 during neutrophil activation, we have employed a unique approach for the quantitative analysis of intracellular antigens which allows simultaneous measurement of total cellular and surface membrane antigen pools.

Stimulation of isolated PMN with N-formyl-Met-Leu-Phe or ionomycin resulted in a mean 7-fold increase in surface CR1 expression within 15 min. Total cellular CR1 decreased by as much as 45% within 15 min, with loss continuing for up to 1 h. Inclusion of NH4Cl during PMN stimulation inhibited the loss of total CR1 without affecting surface CR1 expression. Addition of phenylmethylsulfonyl fluoride inhibited loss of total CR1 and enhanced the stimulus-induced increases in surface CR1.

These data suggest that intracellular degradation of CR1 occurs during stimulation of PMN and may involve proteolysis in an acidic intracellular compartment. Since our experiments were done with isolated PMN in the absence of serum and complement components, this degradation occurred in the absence of C3b, the ligand for CR1. To our knowledge, ligand-independent degradation of a cell surface receptor has not been previously detected.

CR1, the complement C3b/C4b receptor of human neutrophils (PMN), is a membrane glycoprotein of ~205 kDa (1). In addition to complement activation (2, 3), CR1 is important in phagocytosis of soluble and particulate complexes to which C3b has been bound during complement activation (4, 5).

Circulating peripheral blood PMN express only 5500 CR1.

MATERIALS AND METHODS

Antibodies—Monoclonal anti-CR1 antibody of the IgGl subclass produced by clone 3D9 was a gift of Dr. John O'Shea (National Institutes of Health) (9). Irrelevant IgGl monoclonal antibody against rat β-casein was kindly provided by Dr. Charlotte Kaetzel (Case Western Reserve University). Leu-11a anti-PMN Fc receptor, anti-ββ'-microglobulin, and nonspecific IgG1 were purchased as fluorescein isothiocyanate (FITC) conjugates from Becton Dickinson. Affinity-purified FITC-conjugated F(ab')2 goat anti-mouse IgG (Boehringer Mannheim) was used for fixed cells in all experiments and for all cells in experiments which compared fixed and nonfixed cells. Since it gives higher nonspecific binding to fixed cells, (NH4)2SO4-purified FITC-conjugated F(ab')2 goat anti-mouse IgG (Cappel) was not used with fixed PMN, but was employed with nonfixed PMN in the assay for shed CR1.

Neutrophil Isolation and Stimulation—Human PMN were isolated from peripheral blood on discontinuous density gradients of Percoll (Pharmacia LKB Biotechnology Inc.) as described (7). PMN were resuspended at 1-4 x 10^6/ml in Hanks' balanced salt solution without Ca++, Mg++, or phenol red (GIBCO), supplemented with 1 mg/ml of human albumin (Biotype).
gelatin. Stimuli, either 10^{-7} \text{M} \text{ionomycin} (Sigma) and 1.2 \text{mM CaCl}_2 or 10^{-8} \text{M fMLP} (Peninsula Laboratories, Inc.), were added from stock solutions and incubated with PMN at 37 \text{°C} for the indicated times. In some experiments, the following inhibitors or proteases were added concurrently at the indicated final concentrations: 37 \text{mM NiCl}_2, 1 \text{mM phenylmethylsulfonyl fluoride} (Sigma), 0.01 \text{mg/ml soybean trypsin inhibitor} (Sigma), 0.1 \text{mg/ml Eglin C} (Ciba-Geigy), and 0.01 \text{mg/ml trypsin} (Sigma).

**Fixation and Permeabilization**—At the times indicated after addition of stimuli and/or inhibitors, PMN were washed in ice-cold phosphate-buffered saline containing 0.5 \text{mg/ml NaN}_3, and resuspended at 1-4 \times 10^7 \text{ml} in ice-cold periodate/lysine/paraformaldehyde fixative (12) for 20 min. All subsequent steps were at room temperature. PMN were washed with phosphate-buffered saline containing 1 \text{mg/ml ovalbumin} (PBS/ovalbumin) and divided into aliquots of 10^6 cells for staining. One aliquot was permeabilized by washing in PBS/ovalbumin containing 0.4 \text{mg/ml saponin} (Sigma). For these samples, saponin was present throughout staining and subsequent washes. The other aliquot was not permeabilized and was stained in PBS/ovalbumin without saponin.

**Immunofluorescent Staining and Flow Cytometry**—After washing in PBS/ovalbumin with or without saponin, as appropriate (see above), peroxidase-anti-CR1 antibodies were added in excess (0.1 \text{mg/ml}) to each aliquot of 10^6 cells in a total volume of 0.1 ml for 30 min at room temperature. Cells were washed twice with 1.5 ml of PBS/ovalbumin with or without saponin and then reacted with excess (3.4 \text{mg/ml}) FITC-conjugated anti-mouse IgG. After three additional washes, samples were resuspended in phosphate-buffered saline, and fluorescence of 10,000 cells was quantified by flow cytometry exactly as described previously (8). Mean nonspecific background fluorescence was determined using permeabilized cells incubated with FITC conjugate without monoclonal antibody. Control experiments using an excess of irrelevant primary antibody of the same subclass as 3D9 anti-CR1 followed by affinity-purified FITC-conjugated goat anti-mouse IgG had the same mean fluorescence as when the primary antibody was omitted. Mean specific fluorescence of each sample was calculated by subtracting nonspecific background fluorescence from the mean fluorescence of that sample. The specific fluorescence of permeabilized nonstimulated PMN from each experiment served as the maximal fluorescence as when the primary antibody was omitted. The specific fluorescence of each sample was normalized to this maximal control value. Staining was identical for those experiments using nonfixed PMN; but all steps were at 4 \text{°C}, and PBS/ovalbumin contained 0.5 \text{mg/ml NaN}_3.

**Fluorescence Microscopy**—PMN were incubated for 1 h, fixed, permeabilized, and stained for CR1 as above. The cells were mounted in 20 \text{mg/ml n-propyl gallate} in glycerol/phosphate-buffered saline (1:1), pH 9.0, to reduce photobleaching (13).

**Assay for Shed CR1**—To determine if intact CR1 was shed during PMN stimulation, a limiting dilution of anti-CR1 was prepared. A 0.1 mg/ml solution of anti-CR1 was diluted 5-fold with supernatants from various incubation conditions or with intact fMLP-stimulated PMN. These mixtures were incubated at 4 \text{°C} for 45 min, centrifuged at 10,000 \times g for 3 min, and then used to stain aliquots of fresh nonfixed fMLP-stimulated PMN. Binding of anti-CR1 was determined by staining with excess (NH_4)_2SO_4-purified FITC-conjugated goat anti-mouse IgG (Fab'), (250 \text{ mg/ml}) and flow cytometry.

**RESULTS**

**Redistribution and Loss of CR1 During Stimulation of PMN**—We first wished to determine if the total content of CR1 varied as the surface expression increased in response to stimulation. PMN were isolated and resuspended in medium free of serum or C3b. The cells were incubated at 37 \text{°C} with stimuli, and aliquots were removed for staining after 15, 30, and 60 min. The total cellular content of CR1 was measured by indirect immunofluorescence using quantitative flow cytometry of fixed saponin-permeabilized PMN. Surface expression of CR1 was measured on fixed nonpermeabilized PMN. Total CR1 was used to normalize the specific mean fluorescence of all samples within each experiment. This value was identical in freshly isolated PMN (held at 4 \text{°C}) and PMN incubated for up to 60 min at 37 \text{°C} without additional stimuli.

Surface expression of CR1 on PMN stimulated with 10^{-8} M fMLP increased 6.3-fold during the first 15 min of incubation and remained stable over the following 45 min (Fig. 1). In contrast, total CR1 decreased by 15% during the first 15 min of stimulation and fell an additional 15% by 30 min. Thus, even while surface CR1 expression increased, the total cellular content of CR1 decreased.

A similar study of CR1 modulation during ionomycin stimulation (Fig. 2) shows that surface CR1 increased 7.8-fold within the first 15 min, but decreased thereafter, resulting in a net increase of 4.5-fold at 60 min. At 15 min, total CR1 was only slightly greater than surface CR1. After 30 min of ionomycin stimulation, total and surface CR1 were nearly equal, suggesting that the total cellular pool of CR1 was expressed on the surface of PMN as a result of ionomycin stimulation. The late decrease in surface CR1 on ionomycin-stimulated PMN, after the initial increase, may be related to depletion of the intracellular CR1 pool. In contrast, fMLP-stimulated PMN did not express all of their CR1 on the surface (Fig. 1).

**FIG. 1. Effect of fMLP on total and surface CR1 pools.** PMN were incubated with fMLP at 37 \text{°C}, and aliquots were removed after 15, 30, and 60 min of incubation. The PMN were then analyzed for total (■) and surface (□) CR1 as described. The mean of two experiments is shown.

**FIG. 2. Effect of ionomycin on total and surface CR1 pools.** PMN were incubated with ionomycin at 37 \text{°C}, and aliquots were removed after 15, 30, and 80 min of incubation. The PMN were then analyzed for total (■) and surface (□) CR1 as described. The mean of two experiments is shown.
since total CR1 was greater than surface CR1 throughout the period studied. The magnitude of CR1 loss varied slightly between experiments, but within individual experiments, the loss during ionomycin stimulation was always greater than the loss during fMLP stimulation.

Measured CR1 Loss Is Not Due to Fixation or Permeabilization—We considered the possibility that the observed results were due to preferential destruction by the aldehyde fixative of more accessible surface CR1, relative to intracellular CR1 antigen. If this were the case, increased surface expression of CR1 would result in increased loss. To determine whether fixation resulted in loss of detectable surface CR1, we compared surface CR1 detected on fixed nonpermeabilized PMN to surface CR1 detected on identical samples which were incubated and stained in parallel, but not fixed (Fig. 3A). PMN were incubated with or without stimuli for 0-60 min to produce samples with a wide range of surface CR1. Linear regression analysis of fixed and nonfixed aliquots from 41 samples generated a line through the origin with a slope of 0.82 (r = 0.87), indicating an 18% loss of antigen due to fixation. Therefore, although some surface CR1 antigen is not detected after fixation, this loss is too small to account for the 40-60% loss of total CR1 apparent in Figs. 1 and 2.

We then wished to demonstrate that the measured total CR1 was independent of surface expression. Total CR1 detected in fixed permeabilized PMN was plotted as a function of the surface CR1 detected on fixed nonpermeabilized PMN using paired aliquots, as above (Fig. 3B). It is apparent that for any degree of CR1 surface expression, multiple values for total CR1 exist. The points tend to cluster according to the incubation conditions, as shown by the different symbols, suggesting that the extent of total CR1 loss varies according to the type and duration of stimulation. This implies that the loss of CR1 may be a function of the cellular processes activated by various stimuli and is not due to fixation or permeabilization.

Extraction of CR1 during saponin permeabilization of fixed PMN was also considered. Fixed nonpermeabilized cells were stained by indirect immunofluorescence and analyzed by flow cytometry. These same samples were then treated with saponin and re-analyzed by flow cytometry. The specific fluorescence was not substantially altered by saponin treatment, indicating that permeabilization does not release surface CR1 from fixed PMN.

Comparison of CR1, Fc Receptor, and β2-Microglobulin Modulation during PMN Stimulation—To evaluate whether redistribution and loss during PMN activation is specific to CR1, we measured total surface β2-microglobulin and 55-70-kDa PMN Fc receptor in parallel with changes in CR1 during PMN stimulation. The results (Table I) indicate that for all three proteins, only a fraction of the total cellular pool is expressed on the PMN surface. Stimulation with fMLP induced increases in surface CR1, whereas surface expression of both β2-microglobulin and Fc receptor decreased slightly. These decreases in surface expression may reflect internalization, but not degradation, since the total amounts of β2-microglobulin and Fc receptor detected were not altered.

Ionomycin stimulation also reduced surface expression of β2-microglobulin and Fc receptor. Total β2-microglobulin was not affected by ionomycin stimulation. Total Fc receptor decreased during ionomycin stimulation, although only half as much as CR1.

Therefore, the loss and redistribution of CR1 that accompanies increased surface expression represents a novel pattern of modulation of this receptor which is different from that of
TABLE I

Relative fluorescence of total and surface CR1, \(\beta_2\)-microglobulin, and Fc receptor on stimulated and nonstimulated PMN

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>Medium alone</th>
<th>fMLP</th>
<th>Ionomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1 ((n = 14))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.00</td>
<td>0.64 ± 0.04</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Surface</td>
<td>0.10 ± 0.02</td>
<td>0.42 ± 0.03</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>(\beta_2)-Microglobulin ((n = 2))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.00</td>
<td>1.07 ± 0.07</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>Surface</td>
<td>0.31 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>Fc receptor ((n = 2))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.00</td>
<td>0.56 ± 0.04</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>Surface</td>
<td>0.23 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

TABLE II

Inhibition of CR1 loss during PMN stimulation

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>Medium alone</th>
<th>fMLP</th>
<th>Ionomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normalized CR1 fluorescein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fMLP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-NH4Cl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+NH4Cl</td>
<td>0.62 ± 0.06</td>
<td>0.07 ± 0.05</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>Surface CR1</td>
<td>0.43 ± 0.05</td>
<td>0.43 ± 0.04</td>
<td>0.44 ± 0.06</td>
</tr>
<tr>
<td>Ionomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-NH4Cl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+NH4Cl</td>
<td>0.67 ± 0.03</td>
<td>0.03 ± 0.04</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>Surface CR1</td>
<td>0.46 ± 0.02</td>
<td>0.08 ± 0.07</td>
<td>0.07 ± 0.05</td>
</tr>
</tbody>
</table>

\(\beta_2\)-microglobulin and the 55-70-kDa PMN Fc receptor.

Evidence for Intracellular Degradation as the Mechanism of CR1 Loss—Surface receptors on other cell types are known to be internalized and degraded within lysosomes \((14-16)\). Antibody-cross-linked surface CR1 may also be delivered to lysosomes \((17)\). Lysosomal delivery of surface CR1 and other receptors generally depends on ligand binding or cross-linking. However, our experiments utilized isolated PMN in the absence of ligand or any other cross-linking reagent. Despite the absence of cross-linking reagents, we investigated the possibility that the CR1 loss measured might involve internalization and subsequent intracellular degradation.

To examine the role of acidic compartments in CR1 loss, PMN were stimulated in the presence of the acidic triglyceride base NH4Cl, which is known to elevate the pH of acidic PMN organelles \((18)\). Inclusion of NH4Cl inhibited the loss of total CR1 in fMLP-stimulated PMN by 42% \((Table II)\). NH4Cl inhibited total CR1 loss from ionomycin-stimulated PMN by 33%. Surface CR1 expression of fMLP- or ionomycin-stimulated PMN was not affected by NH4Cl addition \((Table II)\). NH4Cl did not alter the surface or total CR1 in nonstimulated PMN. These results suggest that CR1 degradation may occur in an acidic compartment.

To evaluate the importance of proteolytic enzymes in CR1 loss, we stimulated PMN in the presence of phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor which inhibits free access to cell membranes \((19)\). Unlike some other protease inhibitors, PMSF does not alter fMLP binding or uptake \((20)\). PMSF inhibited the loss of total CR1 in fMLP- and ionomycin-stimulated PMN by 87 and 69%, respectively \((Table II)\). In contrast to NH4Cl, which inhibited loss of total CR1 without affecting surface expression, inclusion of PMSF during PMN stimulation allowed additional increments in surface expression of CR1 \((Table II)\). PMSF did not significantly affect surface or total CR1 in nonstimulated PMN. The effect of PMSF on total CR1 was greater than that on surface CR1, indicating that the inhibition of total CR1 loss was not entirely due to increases in surface CR1.

To test the possibility that membrane or secreted proteases degrade CR1 molecules exposed on the cell surface, we stimulated PMN with fMLP in the presence of the cell-impermeant protease inhibitors soybean trypsin inhibitor or Elglin C, a peptide which blocks elastase and cathepsin G activity \((21)\). PMN stimulated in the presence of trypsin were included for comparison. In contrast to PMSF, neither soybean trypsin inhibitor nor Elglin C enhanced fMLP-induced increases in surface expression. Trypsin reduced surface CR1 to less than 10% of the fMLP-stimulated control, confirming the protease sensitivity of CR1 \((22)\). Thus, extracellular proteases do not appear to contribute to CR1 loss, suggesting that the major site of PMSF action is intracellular.

Although the results using NH4Cl and PMSF implicate internalization and intracellular degradation as the mechanism of CR1 loss, we also investigated the possibility that shedding of CR1 from the PMN surface contributed to CR1 loss. This alternative was considered because shedding of CR1 has been demonstrated on osmotically shocked PMN \((23)\), and a soluble plasma form of CR1, possibly shed from blood cells, has been described \((24)\). Supernatants of PMN stimulated with fMLP or ionomycin, which had lost 44 and 56%, respectively, of total CR1 over 1 h, were compared to supernatants of nonstimulated cells. These supernatants were used to make limiting dilutions of anti-CR1. If shed CR1 were present in the supernatants, it would be expected to bind to the anti-CR1 and to inhibit the antibody's ability to bind surface CR1 on fresh fMLP-stimulated PMN. When anti-CR1 was mixed with supernatant from PMN incubated at 37°C without stimuli, with fMLP, or with ionomycin, the amounts bound by surface CR1 on fresh PMN were 90, 86, and 96%, respectively, of that bound with anti-CR1 diluted in buffer alone. Thus, binding of anti-CR1 by supernatants of stimulated PMN was negligible and not significantly different than anti-CR1 binding by supernatants of nonstimulated PMN. When an identical aliquot of anti-CR1 was adsorbed with intact fMLP-stimulated PMN, the amount of anti-CR1 subsequently bound was reduced by 75%, relative to an equivalent amount of anti-CR1 mixed with buffer alone. This verified the sensitivity of the assay. Thus, shedding of intact CR1 does not appear to be a major mechanism of loss during PMN stimulation.

Morphology of CR1 Loss and Redistribution—We used immunofluorescent microscopy to compare the localization of intracellular CR1 in PMN incubated under the conditions described \((Fig. 4)\). The pattern of CR1 staining in fixed permeabilized PMN was similar for PMN incubated at 37°C.
without stimuli or held at 4 °C and was granular throughout the cytoplasm. A negative image of the nuclear lobes was clearly visible, but little surface staining was identifiable (Fig. 4).

Corresponding microscopic examination of fMLP-stimulated cells showed dimmer overall fluorescence which partially outlined the periphery of the cell (Fig. 4). In some cells, faint cytoplasmic staining qualitatively similar to that of nonstimulated PMN was apparent.

Addition of NH₄Cl during PMN stimulation with fMLP significantly altered the pattern of fluorescent staining (Fig. 4). In contrast to PMN stimulated without added NH₄Cl, many bright punctate areas of staining were present in the cytoplasm. These foci seemed smaller than those in nonstimulated PMN and were generally in a single large group which did not define a negative image of the nucleus.

PMSF also altered the distribution of CR1 in fMLP-stimulated PMN (Fig. 4). The overall surface fluorescence was much more uniform than in PMN stimulated with fMLP alone. Additionally, most cells contained a bright cytoplasmic area of staining. These roundish foci were larger than the intracellular patches seen in NH₄Cl-treated or nonstimulated PMN.

Thus, it appears that the initial intracellular pool of CR1 in nonstimulated PMN was largely depleted during stimulation. Inclusion of NH₄Cl or PMSF resulted in the accumulation of intracellular pools which were morphologically distinct from each other and from those in nonstimulated PMN, suggesting that intracellular CR1 exists in at least three distinct compartments, depending on the activation state of the cell.

**DISCUSSION**

We have developed a new approach for the quantitative analysis of cellular antigens which allows concurrent measurement of total cellular and surface membrane antigen. This method may be applicable to the study of other proteins with large intracellular pools and critically regulated surface expression, including the adipocyte glucose transporter (25) and urinary epithelium H⁺ transporters (26). Quantitative analysis of surface expression and proteolysis may also be simplified for proteins like the insulin (16), epidermal growth factor (15), and Fe (14) receptors, which are degraded following ligand-mediated internalization. Although similar information might be obtained by alternative approaches, flow cytometry has the additional advantage of measuring individual cells, thus permitting identification of subpopulations of cells which behave differently. Nonetheless, we are currently working toward verification of our results by an independent approach. The method we describe requires that like CR1, the molecule studied is immunoreactive, accessible to antibody, and not extracted or excessively altered during fixation and permeabilization.

The results indicate that even while chemical stimuli induced 4–8-fold increases in CR1 surface expression, the total cellular pool of CR1 decreased by as much as 60% over 1 h. Since these studies utilized isolated PMN, which are not known to synthesize complement components (27), and neither serum nor complement components were added, this degradation was ligand-independent. Thus, in contrast to other receptors which return to the surface following ligand-independent internalization, CR1 appears to have been degraded.

Our initial finding is corroborated by the data of Changelian et al. (10) who measured total cellular content of CR1 by radioimmunoassay and reported a 15–20% decrease in total cell CR1 during brief phorbol ester stimulation of PMN. The loss they reported is less than the loss we observed, possibly because the duration of their experiments was only 27 min, versus 1 h in this study. Additionally, the cells they used may
have already degraded some CR1 during the activation and increased surface expression induced by their purification procedures (6).

In contrast to CR1, the total cellular β2-microglobulin and Fc receptor remained unchanged after fMLP stimulation. The small decrease in total Fc receptor following ionomycin stimulation suggests that Fc receptor may, in part, follow the same path as CR1. This hypothesis is supported by the observation that cross-linking of either CR1 or Fc receptors by anti-CR1 F(ab')2 or aggregated IgG, respectively, caused co-capping of both receptors (28).

To understand better the redistribution and loss of total CR1, several possible mechanisms were investigated. Although hyperosmotic stress can cause CR1 to be shed from human PMN (23), shedding does not account for the CR1 loss we observed. The apparent lack of involvement of secreted and surface proteases was an expected result since we stimulated PMN in the absence of cytochalasin B, and PMN do not release significant quantities of granular enzymes under these conditions (29).

Inclusion of the weak base NH4Cl during stimulation inhibited the loss of total cellular CR1, consistent with the hypothesis that CR1 is degraded in an acidic intracellular compartment. The fact that surface CR1 expression was not affected by NH4Cl suggests that NH4Cl does not affect CR1 internalization or decrease internalization. Microscopic examination of CR1 in NH4Cl-treated stimulated PMN revealed an intracellular pool which differed morphologically from that in nonstimulated cells and may represent endosomes or immature lysosomes. This would be consistent with the known effects of NH4Cl in preventing acidification and maturation of lysosomes (18, 30).

The membrane-permeant protease inhibitor PMSF also inhibited loss of total cellular CR1 in stimulated PMN. In contrast to NH4Cl, PMSF treatment during stimulation increased surface expression of CR1 above that of controls similarly stimulated without PMSF addition. These data suggest that in cells not treated with protease inhibitors, intracellular degradation may limit the maximum surface expression achievable. This especially seems to be the case with ionomycin stimulation. Alternatively, the activity of a protease or esterase inhibited by PMSF may be necessary for internalization. Most cells stimulated in the presence of PMSF contained a brightly stained intracellular deposit of CR1, possibly representing lysosomes or multivesicular bodies.

The quantitative and morphological data imply that CR1 which is protected from degradation by PMSF may return to the surface membrane and/or be directed to the lysosome. In contrast, neutralization of the endosomal or lysosomal pH with NH4Cl may prevent CR1 from returning to the cell surface. Inhibition of receptor recycling following endosomal pH neutralization has been demonstrated for both epidermal growth factor and transferrin receptors (31, 32). Since pH neutralization also reduces transport from endosomes to lysosomes (30, 33), CR1 protected from degradation by NH4Cl may remain in the endosome. Thus, the differences between the effects of NH4Cl and PMSF are probably due to distinct mechanisms of action since NH4Cl neutralizes the acidic environment, whereas PMSF inhibits proteases, such as cathepsin G and elastase (34, 35), by direct covalent modification without neutralizing the surrounding pH.

Ligand-independent internalization of CR1 occurs in PMN stimulated with activators of protein kinase C, such as phorbol esters or synthetic diacylglycerols (10, 36). Treatment of PMN with phorbol ester or fMLP leads to activation of protein kinase C and induces CR1 phosphorylation in PMN (37). Since protein kinase C activation also results in phosphorylation and ligand-independent internalization of other proteins, such as the epidermal growth factor receptor in epidermoid cells (38, 39), it is possible that the mechanism for ligand-independent internalization of CR1 is similar to that of the epidermal growth factor receptor. However, both epidermal growth factor receptors and macrophage Fc receptors internalized in the absence of ligand are recycled without degradation (14, 38), in contrast to our findings for CR1. CR1 degradation could, in part, be due to its protease sensitivity (22), which may limit the ability of CR1 to survive recycling through acid protease-containing compartments.

The data presented suggest that increased surface expression of CR1 during PMN activation is accompanied by internalization and that internalized CR1 is degraded in an acidic intracellular compartment. Since isolated PMN were incubated without addition of C3b, the CR1 degradation we observed is ligand-independent. The extent to which other receptors are degraded in the absence of ligand remains to be explored.

Acknowledgments—We are indebted to Drs. S. Emanicipator and M. Snider for their critical reviews of the manuscript and Dr. W. Brown for helpful discussion.

REFERENCES
C3b/C4b Receptor Degradation in the Absence of Ligand