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# CR1-receptor recycling in phorbol ester-activated polymorphonuclear leucocytes

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## SUMMARY

Complement-receptor type 1, CR1, which recognizes the C3b cleavage fragment of C3, is present on the membranes of human phagocytic cells, but does not mediate phagocytosis or undergo internalization unless activated by one of a variety of stimuli. Among these stimuli low doses of phorbol esters have been shown to induce a consistently increased expression of CR1, despite apparently continuous receptor internalization. We have studied the fate of internalized receptor–ligand complexes in neutrophils activated with low concentrations of phorbol dibutyrate. In our studies, we followed CR1 with either $^{125}$I-C3b, the physiologic ligand, or with $^{125}$I-Fab fragments of a monoclonal anti-CR1 antibody. We observed rapid internalization of CR1–C3b complexes by PMN treated with 10 ng/ml ($1{\cdot}98 \times 10^{-8}$M) PDBu, consistent in rate and extent with previously reported results using monoclonal antibodies. The fate of the internalized ligand was studied after elution of cell-surface C3b at 0°. Intracellular ligand was externalized in a time- and temperature-dependent fashion, reaching a plateau at 10–15 min. Released C3b was totally TCA precipitable and structurally unaltered, as determined by SDS–PAGE, suggesting that recycling occurs via a prelysosomal predegradative compartment. Loading the cells with chloroquine did not affect this process. A monoclonal anti-CR1 Fab probe behaved in exactly the same manner, suggesting that the recycling of intact ligand–receptor complexes takes place. The possible physiological consequences of this finding are discussed.

## INTRODUCTION

Complement receptor type 1 (CR1) recognizes the C3b fragment of C3. CR1 mediates the attachment of C3b-bearing particles, but not their phagocytosis, by resting polymorphonuclear leucocytes (PMN) and monocytes (Newman & Johnston, 1979; Pommier *et al.*, 1984). Extensive cross-linking of CR1 on unstimulated phagocytes elicits endocytosis, but the unoccupied or monovalently ligated receptor is not demonstrably internalized (Fearon, Kaneko & Thomson, 1981). The number of phagocyte cell-membrane CR1 can be upregulated by a variety of stimuli, including C5a, formyl-methionyl peptides and cell-purification procedures (Berger *et al.*, 1984; Fearon & Collins, 1983). CR1 can also be activated to mediate phagocytosis of C3b-coated particles. Activation can be achieved by interaction of the cell with potentially physiologic stimuli, such as fibronectin, laminin or a T-lymphocyte derived lymphokine, or by non-physiological agents such as phorbol esters, which presumably act through direct activation of protein kinase *C* (Pommier *et al.*, 1984; Bohnsack *et al.*, 1985; Griffin & Griffin, 1980; Pommier *et al.*, 1983; Wright, Craigmyle & Silverstein, 1983; Wright & Silverstein, 1982). Phorbol esters not only render CR1 capable of mediating phagocytosis independently, but also induce the ligand-independent internalization of the receptor in the well-studied case of the human PMN (Changelian *et al.*, 1985; O'Shea *et al.*, 1985a, b). This last effect is temperature, dose, time, intracellular calcium, and microfilament-dependent (O'Shea *et al.*, 1985a, b).

When high doses of phorbol esters are used to stimulate PMN, their cell-surface CR1 receptor numbers decrease dramatically (Changelian *et al.*, 1985; O'Shea *et al.*, 1985a). This finding has been interpreted to indicate that CR1 is downregulated by internalization, followed by accumulation in a latent compartment or by degradation in lysosomes. In contrast, when low doses of phorbol esters are used to stimulate PMN, the cells upregulate their surface CR1 number consistently and for significant periods of time, even when substantial ligand-independent receptor internalization can be demonstrated (Changelian *et al.*, 1985; O'Shea *et al.*, 1985a). The nature of the relationship between CR1 internalization and

Abbreviations: CR1, complement receptor type 1; DMSO, dimethyl sulphoxide; HBSS, Hanks' balanced salt solution; NPGB, *p'*-nitrophenyl-*p*-guanidinobenzoate; PDBu, phorbol 12, 13 dibutyrate; PMN, polymorphonuclear leucocyte; PMSF, phenyl methyl sulphonyl fluoride; TCA, trichloroacetic acid.

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surface expression under such conditions is unclear, as is the fate of the internalized receptor. To examine these issues, we have undertaken studies of the fate of internalized CR1–ligand complexes in low-dose phorbol dibutyrate-treated PMN. Our data suggest that a significant proportion of such complexes are recycled to the cell surface over a brief peroid of time (10–15 min), without passage through the lysosomal compartment.

## MATERIALS AND METHODS

*Reagents and buffers*

The following reagents were purchased: TPCK trypsin, soybean trypsin inhibitor, *p'*-nitrophenyl-*p*-guanidinobenzoate (NPGB), phenylmethylsulphonyl fluoride (PMSF), phorbol dibutyrate (PDBu), dimethyl sulphoxide (DMSO), dibutyl phtalate (Sigma Chemical Co., St Louis, MO) and Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD).

We prepared stocks of 0·1% gelatin in isotonic, 5 mM veronal-buffered saline (GBVS) and 0·1% gelatin in isotonic 5 mM veronal-buffered sucrose (GVBSuc). All the binding and internalization assays were done in isotonic buffer of one-half normal ionic strength, prepared by mixing equal volumes of GBVS and GVBSuc (assay buffer). In order to quantify internalized C3b residues, C3b bound externally to cell-surface receptors was eluted with 0·5 M NaCl, 0·2 M acetate buffer, pH 8·0 (stripping buffer), as described previously (Malbran, Frank & Fries, 1987).

*Proteins*

Human C3 was purified as described elsewhere (Hammer *et al.*, 1981) and labelled using the Iodobeads system (Pierce Chemical Co., Rockford, IL) and $Na^{125}I$ to an average specific activity of 0·21 $\mu$Ci/$\mu$g. C3 was cleaved to C3b by TPCK-trypsin, 1% of C3 by weight, for 2 min at 37°; the reaction was stopped by the addition of a four-fold excess of soybean trypsin inhibitor. The mixture was supplemented with 25 $\mu$M NPGB, 0·1 M sodium acetate, pH 5·6, and solid NaCl to 1 M, and fractionated by sieve chromatography in an AcA-34 column (LKB, Gaithersburg, MD) equilibrated with 1 M NaCl, 0·1 M acetate buffer, pH 5·6, with 25 $\mu$M NPGB (Fearon & Collins, 1983; Fries *et al.*, 1985). C3b-containing fractions were pooled, concentrated by vacuum dialysis, exhaustively dialysed into phosphate-buffered isotonic saline, pH 7·35, and stored in small aliquots at −70° until used.

Monoclonal antibody 1B4 was prepared in our laboratory and was purified from ascites using the octanoic acid method, as described elsewhere (Steinbuch & Audran, 1969). This antibody is a murine IgG1 that is specific for human CR1 as assessed by: (i) inhibition of C3b-dependent rosetting assays; (ii) competitive inhibition of binding of anti-CR1 antibody 3D9 (O'Shea *et al.*, 1985a) to CR1-bearing cells; and (iii) immunoprecipitation of a band or bands from surface-labelled CR1-bearing cells identical to that isolated by other anti-CR1 monoclonals. Fab fragments of antibody 1B4 were prepared by peptic digestion, as described elsewhere (Mage, 1980), and radio-iodinated in the same manner as C3b.

All the proteins used in these experiments were centrifuged for 25 min in an air-driven ultra-centrifuge (Beckman Instruments Inc., Fullerton, CA) at 178,000 *g* immediately before use to remove aggregates. Sucrose density-gradient centrifugation of the C3b pools confirmed the absence of aggregates following this handling.

*Leucocyte isolation*

PMN obtained from normal donors were isolated from heparinized venous blood by room-temperature centrifugation over lymphocyte separation medium cushions, followed by dextran sedimentation of the pellets at room temperature and the hypotonic lysis of residual contaminating erythrocytes (Boyum, 1980).

*Binding and internalization assays*

PMN were preincubated in large volumes of assay buffer for 30 min at 37° in the presence or absence of 10 ng/ml of PDBu ($1{\cdot}98 \times 10^{-8}$M), in order to up-regulate alone (without PDBu) or up-regulate and activate CR1 (with PDBu). The cells were then pelleted and resuspended in a small volume of ice-cold assay buffer containing 25 $\mu$M NPGB (a serine protease inhibitor) at a concentration of $9–15 \times 10^7$ cells/ml. The desired ligand was then added and the mixture incubated for 20 min at 0°, a time shown, in preliminary studies, to permit C3b-binding equilibrium. Non-saturable binding was measured for all points and ligand concentrations in every experiment by the addition of 50–100-fold excess of cold ligand to a parallel, duplicate set of tubes. This value, which includes C3b uptake due to non-receptor-mediated membrane binding and fluid-phase pinocytosis, was subtracted from each total binding determination to yield an estimate of specific binding, and all data cited herein represent specific-binding figures. At the end of the 0° incubation, the cells were warmed to 37°, duplicate aliquots taken over time, and processed as follows. Total-bound counts were determined by pelleting cell samples through 300 $\mu$l of dibutyl phtalate at 10,000 *g* for 90 seconds in a microcentrifuge (Beckman), following which the tube tips were cut and bound radioactivity determined in a gamma counter (Packard Instrument Co., Downers Grove, IL). Internalized ligand was determined by high molar salt elution of surface-bound ligand. The PMN, in 50 $\mu$l of assay buffer, were added to 1 ml of stripping buffer (see above) and incubated at 0° for 10 min. They were then pelleted through 300 $\mu$l of oil and counted as described above. Preliminary experiments demonstrated that this treatment regularly induced the release of 88–91% of externally bound C3b, without affecting cell number or viability as determined by cell count and trypan blue exclusion (Malbran *et al.*, 1987). In experiments requiring elution of surface-bound 1B4 Fab fragments, stripping was accomplished by the published method of O'Shea *et al.* (1985a) utilizing Hanks' balanced salt solution containing 8% fetal calf serum and buffered to pH 3·5.

*Externalization assay*

PMN were allowed to internalize the desired ligand for 15 min at 37°, then washed with HBSS four times at 0°. Preliminary experiments demonstrated that washing four times in ice-cold Hanks' balanced salt solution removed all C3b bound to the cell surface, as assessed by the inability to release further ligand with the high salt-elution protocol detailed above. The cells were then warmed at 37° in normal ionic strength buffer (GVBS) to allow externalization but impede any rebinding of the labelled ligand to cell-surface CR1. Samples were taken over time, incubated in stripping buffer as above, and non-elutable, internal counts were determined. The supernatants were saved, made 10% in trichloracetic acid (TCA) and incubated at 4° overnight. The resultant suspensions were centrifuged at 2000 *g* for 10 min at 4°, split into upper and lower halves, and the fractions counted to

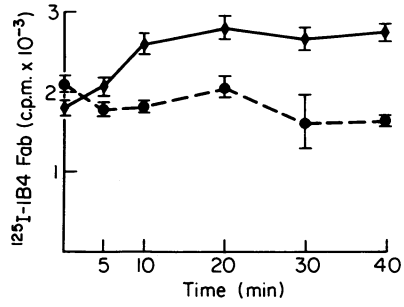


**Figure 1.** Stable up-regulation of PMN membrane CR1 by low-dose PDBu. PMN were treated with 10 ng/ml of PDBu or an equivalent volume of DMSO and incubated at 37°. Samples were taken over time and incubated at 0° with a saturating amount of $^{125}$I-Fab fragments of monoclonal 1B4. Specific binding was determined as in the Materials and Methods. CR1 receptor expression increases over time, reaching a plateau at 10 min and maintaining a steady state for at least 40 min. Control (DMSO treated) cells do not alter their CR1 numbers. (♦) PDBu 10 ng/ml; (●) DMSO.

determine TCA-soluble and -insoluble material. In some experiments a fraction of the TCA-soluble supernatant was diluted with three parts of distilled water to reduce the ionic strength of the solution, and electrophoresed on SDS–PAGE gels under reducing conditions, as described elsewhere (Hames & Rickwood, 1981) to study the molecular structure of the released material.

## RESULTS

### Upregulation and activation of PMN CR1 by low-dose PDBu treatment

Studies by Changelian *et al.* (1985) indicate that low doses of phorbol myristate acetate (PMA) up-regulate the surface expression of CR1 by PMN, whereas higher doses lead to a decrease in membrane-receptor numbers. Using a low dose (10 ng/ml) of phorbol dibutyrate (PDBu), we confirmed the induction of a persistent up-regulation of PMN CR1 at low phorbol ester inputs, as shown in Fig. 1. PMN were preincubated in buffer for 30 min at 37°, and then PDBu 10 ng/ml or an equivalent amount of solvent alone (DMSO) was added. Aliquots were removed over time and cell-surface CR1 assayed by quantifying binding of $^{125}$I-Fab fragments of antibody 1B4 at 0°. The CR1-receptor number is increased on PDBu-treated cells, and this effect is maintained over at least 40–45 min at 37°. Control cells treated with DMSO do not show this effect.

Doses of PDBu identical to those used in the above experiments have been shown by O'Shea *et al.* (1985a), in this laboratory, to induce modest but consistent ligand-dependent internalization of PMN CR1 immediately following addition of the phorbol ester. Using a similar protocol, we were able to demonstrate comparable uptake of $^{125}$I-Fab 1B4 or $^{125}$I-C3b by PMN preincubated with 10 ng/ml PDBu for 30 min at 37° (Fig. 2a). Thus, low-dose PDBu-treated PMN showed the concurrent phenomena of constant and enhanced cell-membrane expression of CR1 (DMSO control 27,858 ± 1841 per cell, PDBu 39,187 ± 2856 per cell, $P < 0.05$, $n = 3$) and apparently continuous internalization of the receptor, at least over a period of 30 min following stimulation. These observations suggested that

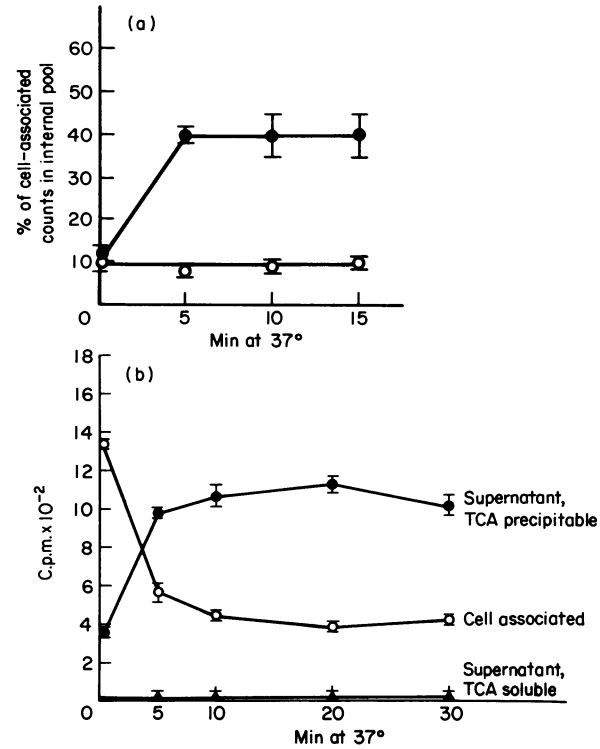


**Figure 2.** (a) Internalization of C3b. PMN were activated with 10 ng/ml PDBu or treated with equivalent amounts of DMSO for 30 min at 37°. The cells were loaded with $^{125}$I-C3b for 20 min at 0° and then transferred to a 37° bath and duplicate aliquots taken over time. Total bound ligand and internalized ligand were determined as in the Materials and Methods. Monomeric $^{125}$I-C3b (●) was internalized by the PDBu-activated PMN but not DMSO control cells (○) in a time- and temperature-dependent fashion, reaching a plateau at 5–10 min. Data represent means ± SE from four separate experiments. (b) Externalization of internalized monomeric C3b. PDBu-activated PMN were allowed to internalize $^{125}$I-monomeric C3b for 15 min at 37°, then washed four times in ice-cold HBSS (a procedure which eliminated all surface-bound ligand as accessed by releasability with stripping buffer). The cells were then rewarmed to 37° in HBSS and samples taken over time and assayed for residual internal C3b, as in the Materials and Methods. Internal C3b was released rapidly, reaching a nadir of 29% of Time 0 values at 20 min.

either the surface-receptor number was kept constant by continuous expression of latent CR1 from an internal pool, or receptor recycling was occurring. Further, the apparent plateau of radioligand accumulation in the intracellular compartment, coupled with evidence of ongoing internalization, implies the concurrent release of internalized ligands. Release might occur via lysosomal degradation of the internalized radioligands or through recycling and dissociation of intact receptor–ligand complexes. In order to address these questions we performed the following experiments.

### Internalization and subsequent recycling of CR1–C3b complexes

PMN were stimulated for 30 min at 37° with 10 ng/ml PDBu, chilled to 0°, and then allowed to bind $^{125}$I-C3b in assay buffer at 0°. Without washing, the cells were then re-warmed to 37° and samples taken over time and processed as described above.

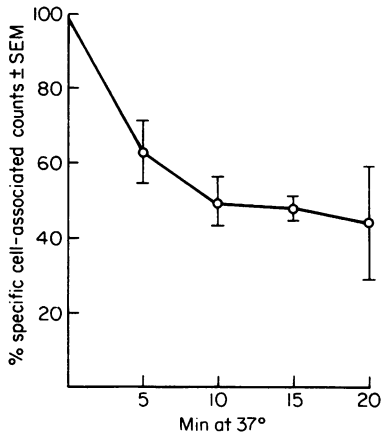


**Figure 3.** Time-course of externalization of C3b by neutrophils. Data from four separate experiments performed as described in Fig. 2b are displayed as mean values ± SEM. The ligand was invariably present as a TCA-precipitable form in the supernatant.

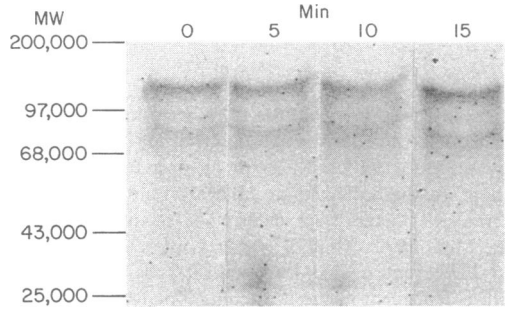


**Figure 4.** SDS–PAGE electrophoresis and autoradiography of released material. The $^{125}$I-C3b recovered from cell supernatants at 0, 5, 10 and 15 min after warming to initiate externalization showed intact $\alpha'$- and $\beta$-chains with apparent MW of 115,000 and 75,000, respectively. No low molecular weight degradation products are noted.

Figure 2a shows the results of these experiments. Nine to twelve percent of cell-bound C3b was not releasable by our stripping conditions, even when no 37° incubation occurred (0 time). The non-releasable fraction increased to 40% of the total cell-bound ligand with warming to 37°, reaching plateau values by 5 min. Cells incubated with buffer or DMSO in buffer demonstrated no such increase. To study the fate of internalized $^{125}$I-C3b we washed PMN, which had internalized $^{125}$I-C3b for 15 min at 37°, four times in ice-cold HBSS. This procedure resulted in release of external ligand comparable to that achieved by stripping buffer. Such washed PMN could then be rewarmed to 37° and the fate of the internalized ligand pool followed, as outlined in the Materials and Methods. As can be appreciated from Fig. 2b, the cell-associated counts rapidly decreased over time, reaching a stable value at 10–20 min. The $^{125}$I-C3b was released into the medium with reciprocal kinetics as completely TCA-precipitable material. Figure 3 shows composite kinetic data from four separate experiments evaluating ligand release from the internalized pool. Since PMN from different donors express different numbers of receptors on their surface, the data are expressed as

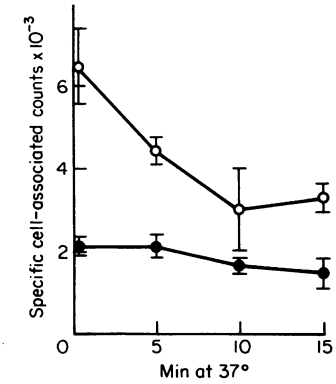


**Figure 5.** Externalization of internalized $^{125}$I-Fab anti-CR1. PMN were allowed to internalize $^{125}$I-Fab anti-CR1 1B4, then washed extensively with ice-cold HBSS and rewarmed to 37°. Samples were taken over time and residual internal ligand was determined as described elsewhere (O'Shea *et al.*, 1985a). The cell-associated radioactivity declined over time in the same manner as $^{125}$I-C3b, the physiological ligand of CR1, in PDBu-activated PMN (open symbols). Control cells treated with equivalent volumes of DMSO did not internalize the ligand and did not show significant release (closed symbols). Data shown are means and ranges of duplicate determinations.

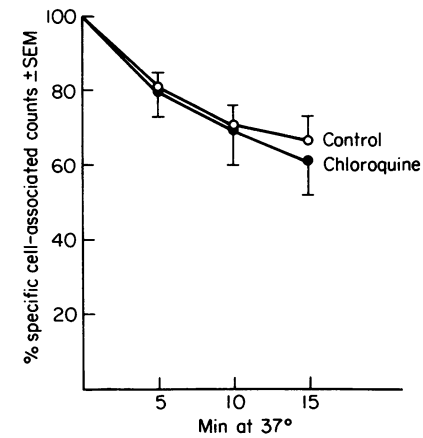


**Figure 6.** Effect of chloroquine on release of internalized $^{125}$I-C3b. PMN were activated and allowed to internalize the ligand in the continuous presence of 1 mM chloroquine. They were then washed at 0° and rewarmed to 37° in chloroquine containing HBSS, and the externalization phase studied. Data represent means and SEM from two separate studies. No significant difference in externalization between control and chloroquine-treated PMN was found.

a percentage of the total amount of C3b internalized at Time 0. Released $^{125}$I-C3b was invariably entirely TCA-insoluble, and SDS–PAGE followed by autoradiography of the released material revealed intact $\alpha'$- and $\beta$-chain structure (Fig. 4). The lack of degradation of C3b was not an artifact of the NPGB present in the media, since the same results were reproduced in several experiments in which this protease inhibitor was not utilized. Taken together, these data suggest that $^{125}$I-C3b that was internalized and released did not pass through a lysosomal compartment. Since published data on the internalization of CR1 were obtained using monoclonal anti-receptor antibodies,

we assessed whether anti-receptor Fab fragments were recycled to the cell surface in the same way as the physiological ligand, C3b. As shown in Fig. 5, our findings could be reproduced using $^{125}$I-FAb 1B4. Equivalent internalization and subsequent release of $^{125}$I-1B4 Fab was observed in reduced ionic strength assay buffer, or in normal ionic strength buffer as described by O'Shea *et al.* (1985a), in both cases without NPGB. In addition, it can be appreciated from the same figure that control cells treated with DMSO, which did not internalize the probe, also did not demonstrate the release phenomenon.

### Effect of chloroquine on the externalization mechanism

The rapid kinetics of release and the intact state of released C3b suggested that recycling was proceeding via a pre-lysosomal compartment. Since it has been shown in many ligand–receptor systems that lysosomotropic agents such as chloroquine are able to interrupt receptor recycling and ligand release to lysosomes, (Chang & Kullberg, 1984; Tietze, Schlesinger & Stahl, 1980) we studied the effect of chloroquine on the externalization of C3b. PMN were PDBu activated, equilibrated with $^{125}$I-C3b, allowed to internalize, washed and warmed to permit externalization, all continuously in the presence of 1 mM chloroquine, a concentration previously shown to modify PMN lysosomal acidification (Klempner & Styrt, 1983). Figure 6 demonstrates that chloroquine did not alter C3b release with respect to simultaneous controls. This finding reinforces the hypothesis that a prelysosomal and probably non-acidic vesicle compartment accommodates the majority of recycling CR1 in this system.

## DISCUSSION

CR1 has been shown to be internalized in a ligand-independent way when PMN are stimulated with phorbol esters (Changelian *et al.*, 1985). This process appears in parallel with the acquired capacity of activated PMN to phagocytose particles coated with C3b as the sole opsonin (O'Shea *et al.*, 1985a). The relationship between these observations is unclear. The ligand-independent internalization of CR1 demonstrates a plateau after 5 or 10 min (Changelian *et al.*, 1985; O'Shea *et al.*, 1985a). Although CR1 is continuously internalized, studies of cell-membrane CR1 indicate stable up-regulation of cell-surface receptor numbers at low phorbol ester doses. These phenomena could be explained by two different hypotheses: (i) the internalized receptor is routed directly to a lysosomal compartment, where ligand is very rapidly degraded and then released from the cell, producing an apparent plateau of uptake, or (ii) a large part of the receptor and its ligand are recycled to the surface of the cell. Either hypothesis could accommodate the concurrent presence of a latent internal CR1 pool which replenished some portion of internalized receptors. To examine these hypotheses, a simple system to distinguish cell-bound versus cell-internalized C3b has been developed. C3b bound to its receptor on the external surface of the cell is removed by increasing the ionic strength of the medium (a manoeuvre to which the C3b–CR1 interaction is highly sensitive; Fries *et al.*, 1985), whereas internalized ligand is not displaced. To complement this approach, we also studied internal and external distribution of an anti-CR1 monoclonal Fab by methods described previously (O'Shea *et al.*, 1985a).

The internalization of monomeric C3b followed the pattern of previous studies performed in our laboratory with monoclonal anti-CR1 Fab fragments (Changelian *et al.*, 1985; O'Shea *et al.*, 1985a, b). When cells that had internalized C3b were washed free of external ligand and then rewarmed to 37°, the cell-associated radioactivity decreased rapidly, again reaching a stable value at 10–20 min. The released material retrieved from the supernatants was precipitated totally by 10% TCA and structurally intact C3b by SDS–PAGE, suggesting that the ligand was not degraded by the cells and was passing through a prelysosomal compartment. Furthermore, the kinetics of release were not affected by loading the cells with chloroquine. Very similar externalization data were obtained using a different and non-physiological probe for CR1, monoclonal 1B4 anti-CR1 Fab fragments. Since the 1B4 Fab–CR1 complexes are unlikely to be susceptible to the same dissociative forces as the physiologically ligated C3b–CR1 complexes, the congruence of the Fab fragment and C3b results strongly suggests that our data reflect the recycling of ligand–receptor complexes and not the release of free ligand alone. Similarly, the artifactual appearance of 'internalization', induced by modulation of CR1 affinity for C3b, would be unlikely to be reproduced by a non-physiological probe such as 1B4. The proportion of internal counts externalized and released varied from 40 to 70% at 15–20 min. It should be noted, however, that a proportion of cell-bound counts were not strippable—even when the PMN were not stimulated with PDBu or warmed to 37°. When this irriducable background was disregarded, evaluable externalization exceeded 60% of internal counts in every case. This finding exactly parallels observations in several other receptor systems, such as murine macrophage Fc$\gamma$ and mannose receptors and skin fibroblast low-density lipoprotein receptors, which recycle rapidly through a non-degrading compartment when tagged with monovalent ligand (Mellman, Plutner & Ukkonen, 1984; Tietze, Schlesinger & Stahl, 1982; Greenspan & St Clair, 1984).

In summary, we have shown that a majority of CR1–monovalent ligand complexes internalized by low-dose PDBu-treated PMN are returned to the cell surface rapidly and apparently without delivery to lysosomes. Although our data do not address the possible contribution to replenishment of cell-surface CR1 by a latent internal pool, recycling of internalized receptor is clearly of sufficient magnitude to play a role in maintaining cell-surface CR1 numbers in activated PMN. To the extent that phorbol ester activation mimics the status of cells which are migrating to sites of inflammation, such recycling would allow PMN CR1 to manifest phagocytic activity yet avoid receptor down-regulation, despite the presence of soluble monomeric C3 and C4 cleavage products. C3 is the most abundant complement component in human serum, and C3 cleavage products may be generated in significant concentrations at sites of inflammation. Since a majority of these molecules will be released as soluble products rather than bound to a significant target of phagocyte activity (e.g. a bacterium) it is clearly advantageous for PMN to release any internalized monomeric C3b and recycle the involved CR1.

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