Complement Receptor 3 (CD11b/CD18) Mediates Type I and Type II Phagocytosis During Nonopsonic and Opsonic Phagocytosis, Respectively

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Two types of opsonic phagocytosis have been defined depending on the receptor engaged: FcγRs mediate type I phagocytosis of IgG-coated particles; complement receptor 3 (CR3) mediates type II phagocytosis of complement-coated particles. In addition to opsonic phagocytosis, CR3 also mediates nonopsonic phagocytosis of zymosan (Z) and *Mycobacterium kansasii* through engagement of distinct sites. Using Chinese hamster ovary cells stably expressing human CR3, we studied CR3-mediated ingestion of nonopsonized particles, Z, or *M. kansasii*, compared with opsonized zymosan (OZ). We show that 1) while OZ sinks into cells, Z is engulfed by pseudopodia as visualized by electron microscopy; 2) in contrast to OZ, nonopsonic phagocytosis of Z and *M. kansasii* depends on Rac and Cdc42 but not on Rho activity; and 3) CR3-mediated phagocytosis of Z depends on the kinase activity of the Src family tyrosine kinase Hck, while OZ internalization does not. Therefore, CR3 mediates type I phagocytosis under nonopsonic conditions and type II under opsonic conditions. This is the first evidence that a single receptor can mediate both types of phagocytosis depending on the ligand used. The Journal of Immunology, 2002, 169: 2003–2009.

In addition to opsonic phagocytosis, microorganisms can be ingested independently of the presence of opsonins at their surface. This type of phagocytosis is particularly important to eradicate infections occurring at sites that are poor in serum opsonins such as the lung. Several receptors have been involved in ingestion of diverse groups of microbes such as mannose receptor, scavenger receptors, CD14, or β-glucan receptor but also CR3 (8–11). In addition to its role in opsonic phagocytosis and cell adhesion, CR3 actually serves in the nonopsonic recognition of microbes by interacting directly with molecules on their surface (12–17), but the type of phagocytosis concerned has not been defined.

CR3 is an heterodimeric transmembrane glycoprotein, belonging to the β2-integrin family, consisting of CD11b noncovalently associated with CD18 (reviewed in Refs. 18, 19, and 20). Distinct functional domains have been identified in the extracellular portion of the CD11b subunit of CR3 (21–25); the I- or A-domain is essential for binding and phagocytosis of C3bi-coated particles (22, 25), and the lectin domain, located C-terminal to the I-domain (23), is responsible for the nonopsonic binding properties of CR3 (9, 21). Moreover, we have recently shown that under nonopsonic conditions, phagocytosis of zymosan (Z) and *Mycobacterium kansasi* involves distinct molecular determinants of the receptor, and that nonopsonic phagocytosis of *M. kansasii*, in contrast to Z, occurs independently of the C-terminal lectin site (26).

The purpose of this study is to define whether mechanisms involved in nonopsonic phagocytosis mediated by CR3 are distinct from those involved in C3bi-mediated phagocytosis. In addition, we compared the nonopsonic internalization of Z and *M. kansasii* which bind to distinct CR3 sites (26). A limiting factor to studying a single phagocytic receptor in phagocytes is that they express several phagocytic receptors which often cooperate to engulf IgG (FcγRs), C3bi-coated particles (CRs), or complex microorganisms (8, 27–31). For instance, Z particles are internalized by mannose receptor, CR3, and β-glucan receptor (9, 32, 33). C3bi-coated particles by CR3 and CR1 (34), and mycobacteria by mannose receptor, CR3, GPI-anchored proteins such as CD14, and scavenger...
receptor (11, 26, 31, 35–38). Therefore, it is very difficult to evaluate the unique contribution of CR3 during phagocytosis of these particles in phagocytes. To circumvent this problem, we used nonphagocytic Chinese hamster ovary (CHO) cells stably expressing the human CR3 (CHO-CR3 cells) (11, 22). Although CHO cells are devoid of phagocytic receptors, they express receptors previously shown to interact with mycobacteria and zymosan such as Toll-like receptors (TLRs), especially TLR2 and TLR4 (39–42). However, in CHO cells TLR2 is nonfunctional (40) and TLR4 is unable to trigger cell activation in response to mycobacteria (41). Therefore, TLRs are unlikely to contribute to binding of Z, opsonized zymosan (OZ), or mycobacteria in CHO-CR3 cells.

Using this cellular model, we could specifically study CR3-mediated opsonic and nonopsonic phagocytosis, and we show that distinct phagocytic mechanisms take place during ingestion through distinct binding sites of CR3.

**Materials and Methods**

**DNA constructs and reagents**

Constructions of pSPHck and pSPHck ⋆ in fusion with GFP have been described (43, 44). Expression vectors encoding Myc-tagged dominant negative forms of Rac and Cdc42 were kindly provided by A. Hall (University of London, London, U.K.) (3). Expression vector encoding FcγRIIa and anti-FcγRIIa IV.3 mAbs were nicely supplied by C. Sautes-Fridman (Institut National de la Santé et de la Recherche Médicale, Unité 255, Paris, France) (45). CR3 exoenzyme from Clostridium botulinum (46) was a generous gift from P. Boquet (Institut National de la Santé et de la Recherche Médicale, Unité 452, Nice, France), RPMI 1640, α-MEM, t-glutamine, and antibiotics were purchased from Life Technologies (Cergy Pontoise, France). Antimycobacteria rabbit Camelia Abs have been previously described (26). FITC- and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit, anti-mouse Abs, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). Anti-myc Abs 9E10 were purchased from Invitrogen (Cergy Pontoise, France).

**Mycobacteria and zymosan particles**

*M. kansasii* (ATCC 124478; American Type Culture Collection, Manassas, VA) was grown, prepared, and stained with FITC as previously described (47). Z was stained with FITC (47) and used, OZ or not, as described (48). IgG-opsonized Z was prepared by incubating particles in suspension in PBS with human IgG (13 mg/ml, 30 min at 37°C) and washed as described (48). The number of particles or bacteria was counted in a Thoma’s chamber.

**Cell culture and transfection**

CHO cells that express human CR3 in its active state (CHO-CR3) were obtained from T. A. Springer (Harvard Medical School, Boston, MA) (22). A subclone of CHO-CR3 cells which expresses CD11b/CD18 at a high rate (11) was used in this study as previously described (26). When indicated, CHO cells were transfected by the DNA/calcium precipitation method as described (49). Human neutrophils and monocytes were isolated from blood of healthy donors as previously described (48).

**Phagocytosis assay**

To remove seric proteins, cells were washed three times and incubated for 30 min at 37°C in serum-free medium. FITC-labeled Z particles or mycobacteria were then added at a multiplicity of infection of 50:1. Cells were then extensively washed with α-MEM medium and fixed in paraformaldehyde (26). To exclusively quantify phagocytosis and not binding, extracellular Z and mycobacteria were stained as described (26). Contact between cells and nonopsonized particles was maintained overnight as previously described (11). This allowed us to reach a percentage of cells having ingested at least one particle sufficiently high (31.0 ± 2.9% (n = 5) for Z and 29.6 ± 7.0% (n = 5) for *M. kansasii*) to quantify inhibitory effects more easily. Because phagocytosis of OZ is much more efficient than that of nonopsonized Z, it was added to cells 3 h before the end of the overnight incubation. The percentage of OZ phagocytosis was 49.2 ± 4.2% (n = 5). Extracellular OZ was stained using the protocol described for Z and mycobacteria (26).

In experiments with transfected cells, transient expression of Cdc42 or Rac in CHO-CR3 cells was detected by anti-myc Abs and revealed by tetramethylrhodamine isothiocyanate-conjugated secondary Abs; transient expression of Hck was revealed by green fluorescent protein (GFP) fluorescence, and FcγRIIa-positive cells by anti-FcγRIIa Abs revealed by FITC-conjugated secondary Abs. Phagocytosis of Z, OZ, or *M. kansasii* by fluorescent cells was measured and compared with mock-transfected cells. The percentage of transfected cells was 20–40% and these cells were analyzed for Z, OZ, or *M. kansasii* phagocytosis by counting at least 100 transfected cells per coverslip. When indicated, cells were pretreated with 10 μM pyrazolo pyrimidine (PP)1 before the addition of Z or OZ to inhibit the Src protein tyrosine kinases (FTKs) expressed in CHO-CR3 cells. In some other experiments, cells were incubated for 24 h with 50 μg/ml RC3 exoenzyme to inhibit Rho by ADP-ribosylation. The toxin was added 8 h before the addition of Z or OZ particles. Contact between cells and particles was maintained for 16 h with both types of particles in the presence of the toxin. Coverslips were viewed using a Leica DM-RB fluorescence microscope (Leica Microsystems, Rueil-Malmaison, France) or a Leica DMRE equipped with the TCS-SF2 confocal scanning system.

Data are presented as the mean ± SEM of the indicated number of experiments performed in duplicate. Statistical analysis was determined using unpaired Student’s *t* test (*p* < 0.05; **p** < 0.01; ***p*** < 0.005).

**Lactate dehydrogenase (LDH) measurement**

Cell viability was assessed by measurement of the release of the cytosolic enzyme LDH using the colorimetric assay kit from Boehringer Mannheim (Meylan, France; Ref. 49).

**Electron microscopy**

To optimize the experiments and obtain a maximal number of nascent and early phagosomes, the cell/particle contact was maintained 7 h with Z and 30 min with OZ in CHO-CR3 cells and for 5 min and 10 min with Z in human neutrophils and monocytes, respectively. Adherent CHO-CR3 cells were washed free of serum and divided into two pools which were incubated for 7 h. At time 0, zymosan was added in pool 1 and OZ was added 6.5 h later in pool 2. CHO-CR3 cells were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h. After fixation, cells were washed in cacodylate buffer, postfixed for 4 h at 0°C with 2% osmium tetroxide, dehydrated, and embedded in Epon 812 (EPON, Fluka, Switzerland) (50). Examination was performed with a Philips EM 301 electron microscope (Eindhoven, The Netherlands). Adherent neutrophils were incubated for 15 min at 4°C with Z in MEM buffered with 20 mM HEPES, pH 7.4 (MEM-HEPES), washed two times with MEM-HEPES, and incubated for 5 min at 37°C. At the end of the incubation period, cells were fixed as described for CHO-CR3 cells. Adherent monocytes were incubated for 10 min with Z in RPMI 1640 at 37°C under 5% CO2 atmosphere, washed two times with RPMI 1640 at 4°C and fixed as described above.

**Results and Discussion**

To examine whether CR3 mediates phagocytosis through distinct mechanisms, we first verified that CHO cells can carry out the two types of opsonic phagocytosis described to date, those mediated by CR3− (type II) and FcγR (type I). To this aim, CHO-CR3 cells were incubated with zymosan (membrane particles of *Saccharomyces cerevisiae*, Z) opsonized in human serum (OZ). After 3 h of coinubation, 49.2 ± 4.2% (n = 3) of the cells had internalized OZ particles. This uptake was mediated by CR3 since it was inhibited by anti-CR3 Abs (data not shown) and only 6.5 ± 4.9% (n = 6) of wild-type CHO cells ingested OZ. Next, CR3 CHO cells were transiently transfected with FcγRIIa. Transfected cells were coinubated with Z coated with purified human IgG for 3 h and 34.9% (n = 1) of the cells internalized IgG-opsonized Z. These results suggest that CHO cells possess the appropriate machinery to mediate both type I and type II phagocytosis.

Zymosan is also a nonopsonic ligand of CR3 (9) and we have previously reported that phagocytosis of Z by CHO-CR3 cells is inhibited by anti-CR3 Abs while wild-type CHO cells did not significantly ingest Z particles (26). So, using Z and OZ we could examine nonopsonic and opsonic phagocytosis mechanisms mediated by CR3 in CHO cells by electron microscopy. As shown in Fig. 1A, particles of OZ sank into the cells as previously described (4, 5). In contrast, nonopsonized Z was engulfed by pseudopodia...
FIGURE 1. CR3-mediated phagocytosis of opsonized and nonopsonized zymosan particles occurs through different mechanisms. Transmission electron micrographs of CHO-CR3 cells incubated with OZ (A) or Z (B). Cells ingesting Z show protrusion of pseudopodia from the cell surface, which initiate the contact with particles (B, a–c) in an early step of the phagocytic process and then engulf Z particles (B, d–j). In contrast, OZ sink into the cell (A, a–e) generating little (Ac) or no membrane protrusions (A, a and b) at early time points. Transmission electron micrographs of human neutrophils (C) and monocytes (D) incubated with Z also show pseudopodia extension. Bars, 1 μm. Galleries of representative pictures are shown.
(Fig. 1B) as previously observed for IgG-coated particles phagocytosed through FcγRs (4, 5). This shows that different mechanisms are probably operating in phagocytosing CHO-CR3 cells and suggests that the nonopsonic CR3-mediated phagocytic process is a type I phagocytosis.

We next examined which type of phagocytosis was taking place during Z ingestion by human neutrophils and monocytes freshly isolated from human blood. We observed that both cell types internalized Z by pseudopodia-mediated process (Fig. 1, C and D, respectively) as observed in CR3-CHO cells. These phagocytes do not express the mannose receptor which participates in phagocytosis of Z in macrophages (32, 51). Although we have previously reported that CR3 plays a major role in neutrophils and macrophages to ingest Z (26, 38), we cannot exclude that other receptors such as the new β-glucan receptor (52), could, at least partially, participate in this process. Besides, it is noteworthy that pseudopods protruding from the surface of professional phagocytes look longer than those protruding from the surface of CHO-CR3 cells. This could be due to the fact that CHO cells are not genuine phagocytes.

Types I and II phagocytosis share a common property because both are affected by actin-depolymerizing drugs. However, the intracellular distribution pattern of actin-binding proteins can be different depending on the particle ingested. When mice macrophages ingest C3bi-opsonized Z through CRs, then F-actin, vinculin, α-actinin, and paxillin are recruited to phagosomes, where they are distributed as patches as observed by confocal microscopy (5). In contrast, when IgG-coated beads are internalized through FcγRs, those proteins are uniformly distributed (5). To determine whether these differences do exist also between opsonic or nonopsonic CR3-mediated phagocytosis, CHO-CR3 cells were examined by fluorescence microscopy using rhodamine-coupled phalloidin to stain actin filaments (Fig. 2) or Abs directed against two actin-associated proteins, vinculin and paxillin (data not shown). Distribution of actin was similar on both phagocytic cups (nascent phagosomes) observed by epifluorescence microscopy (Fig. 2A) and phagosomes containing Z or OZ observed by confocal microscopy (Fig. 2B) or epifluorescence microscopy (data not shown). As our results do not match the actin patterns previously observed during phagocytosis of IgG and complement-opsonized particles (5), this approach cannot be used to further distinguish between opsonic and nonopsonic phagocytosis mediated by CR3 in CHO-CR3 cells.

To establish the molecular bases of the ultrastructural differences described above, we next analyzed the relative role of Rho-family proteins in opsonic and nonopsonic phagocytosis mediated by CR3, because they have been differentially involved in types I and II phagocytosis (3, 53). For this purpose, CHO-CR3 cells were transiently transfected with dominant-negative forms of Rac (RacN17) or Cdc42 (Cdc42N17). In both cases, ingestion of Z was markedly inhibited (Fig. 3A) whereas phagocytosis of OZ was not altered (Fig. 3B), as previously reported (3). Expression of these dominant-negative Rho family proteins did not affect cell viability (data not shown). Mycobacteria are also internalized by CR3 under nonopsonic conditions (11, 26). We thus examined whether Rho family GTPases could be involved in this process. We observed that transient expression of RacN17 or Cdc42N17 also inhibited nonopsonic phagocytosis of M. kansasii (Fig. 3C). These results indicate that CR3-mediated nonopsonic phagocytosis depends on Rac and Cdc42 in contrast to opsonic phagocytosis, and further indicate that CR3-mediated nonopsonic phagocytosis is a type I phagocytic process. RhoA has been shown to be involved in type II, but not type I, phagocytosis (3). To examine its

**FIGURE 2.** Filamentous actin (F-actin) is similarly enriched on phagocytic cups (A) and early phagosomes (B) containing zymosan or opsonized zymosan. Phagocytosis of FITC-labeled Z for 2 (A) and 7 (B) h or opsonized FITC-Z for 30 min (A) and 1 h (B) is shown. F-actin was visualized using rhodamine-phalloidin by fluorescence microscopy (A) and confocal microscopy (B). A Arrowheads show F-actin-enriched phagocytic cups and the corresponding FITC-Z particles. Pictures representative of three independent experiments are shown. B, Each panel represents a single section of confocal microscopy.

**FIGURE 3.** RacN17 and Cdc42N17 expression inhibits phagocytosis of Z (A) and OZ (B). M. kansasii (C). A, CHO-CR3 cells were transiently transfected with RacN17 or Cdc42N17 and incubated for 24 h. Inhibition of phagocytosis was determined with fluorescence microscopy using rhodamine-coupled phalloidin to stain actin filaments (Fig. 3A) or Abs directed against two actin-associated proteins, vinculin and paxillin (data not shown). Distribution of actin was similar on both phagocytic cups (nascent phagosomes) observed by epifluorescence microscopy (Fig. 3A) and phagosomes containing Z or OZ observed by confocal microscopy (Fig. 2B) or epifluorescence microscopy (data not shown). B, Inhibition of phagocytosis of OZ was not altered (Fig. 3B), as previously reported (3). **C, M. kansasii** was also internalized by CR3 under nonopsonic conditions (11, 26). Expression of these dominant-negative Rho family proteins did not affect cell viability (data not shown). We thus examined whether Rho family GTPases could be involved in this process. We observed that transient expression of RacN17 or Cdc42N17 also inhibited nonopsonic phagocytosis of M. kansasii (Fig. 3C). These results indicate that CR3-mediated nonopsonic phagocytosis depends on Rac and Cdc42 in contrast to opsonic phagocytosis, and further indicate that CR3-mediated nonopsonic phagocytosis is a type I phagocytic process. RhoA has been shown to be involved in type II, but not type I, phagocytosis (3). To examine its
role in CR3-mediated phagocytosis of Z and OZ, CHO-CR3 cells were incubated in the presence of C3 exoenzyme. This toxin ADP ribosylates several Rho GTPases and disrupts their interaction with downstream effectors. This effect is illustrated by the disappearance of stress fibers (Fig. 4A) as previously described (43, 54). Fig. 4B shows that OZ phagocytosis was drastically inhibited by the toxin, whereas Z internalization was not altered. These results thus confirm that CR3-mediated nonopsonic internalization is a type I phagocytosis.

PTKs of the Src family have been implicated in the signaling of FcγRs, and CR3 when playing its integrin role (6, 7, 43, 55, 56). So, we examined the role played by Src PTKs during CR3-mediated phagocytosis using PP1, a specific Src PTK inhibitor. A marked difference was observed between phagocytosis of nonopsonized and opsonized particles because Z and M. kansasii were inhibited by 73.0 ± 9.6% and 61.5 ± 10.7%, respectively (mean ± SEM of five to seven separate experiments) and OZ by 27.9 ± 9.3% (mean ± SEM of four separate experiments). However, measurement of LDH release showed a cytotoxic effect of PP1 (25.2 ± 0.9% of LDH release over control cells, mean ± SEM of four separate experiments). Because the toxic effect of PP1 could account for the small inhibitory effect observed with OZ ingestion, a second approach was undertaken to study the role of Src PTKs in the signaling of CR3.

Src PTKs have been described as molecules able to transduce signals not only through their classical kinase function, but also through their Src homology (SH2)- and SH3-mediated adaptor function in a kinase-independent manner (44, 57). Overexpression of dominant-negative forms of Src PTKs which lack their kinase activity but keep their SH2- and SH3-dependent adaptor role has been useful to study their functions (reviewed in Ref. 58). Recently, using a dominant-negative form of the Src-family member p59Hck (p59Hckdn) expressed in HeLa cells, we demonstrated that p59Hck is involved in actin cytoskeleton rearrangement by activation of the Cdc42/Rac pathway leading to formation of membrane protrusions necessary for particles engulfment (44). So, we examined whether p59Hckdn could affect CR3-mediated phagocytosis, and we show that phagocytosis of Z or OZ was strongly affected by p59Hckdn (Fig. 5). In contrast, transfection of the wild-type p59Hck (p59Hck WT) strongly inhibited phagocytosis of OZ.
but not of Z (Fig. 5). These results indicate that both opsonic and nonopsonic phagocytosis are dependent on Hck but two distinct mechanisms are involved: first, the kinase activity of the endogenous Src PTKs expressed in CHO-CR3 cells is involved in phagocytosis of Z. Indeed, PPI inhibited its ingestion and overexpression of p59Hck WT that chelates the substrates of endogenous Src PTKs also inhibited phagocytosis. As expected, overexpression of p59Hck WT which is enzymatically functional did not affect the phagocytic process. Second, the kinase activity of endogenous Src PTKs is not involved in phagocytosis of OZ. Indeed, PPI exhibited a low inhibitory effect which was probably due to its cytotoxic effect. Consequently, we expected that p59Hck WT, which does not have any kinase activity, would not inhibit phagocytosis of OZ. However, it inhibited phagocytosis of OZ suggesting that it works as a scavenger of the nonsubstrate effectors of the endogenous Src PTKs, a role also played by p59Hck WT which equally inhibited phagocytosis of OZ when overexpressed in CHO-CR3 cells (Fig. 5). To conclude on Src PTKs, we report that although they are involved in phagocytosis of Z and OZ, their kinase activity is involved in type I phagocytosis whereas their adaptor function is involved in type II phagocytosis, further distinguishing the two types of particle ingestion.

In conclusion, CR3 is able to perform phagocytosis of nonopsonized or complement-opsonized particles through distinct mechanisms. Phagocytosis of Z is similar to type I phagocytosis, it involves pseudopodial extensions, Rac, Cdc42, but not RhoA, and is regulated by the kinase activity of Hck. Phagocytosis of OZ is similar to type II, it is characterized by the sinking of particles into the cells, it is Rac- and Cdc42-independent, but RhoA-dependent, and involves Src PTKs for their adaptor function but not their kinase activity. This suggests that CR3 triggers different intracellular signals which mediate distinct phagocytic processes. Accordingly, it has recently been described that CR3 can trigger different signaling pathways leading to delayed or enhanced apoptosis in neutrophils (59). In addition, β2 integrins use different signaling mechanisms to support migration and other integrin-mediated effector functions in neutrophils (60). We have previously shown that Z and OZ bind to distinct sites on CR3 (26). Depending on the binding site involved, CR3 could take different conformations leading to activation of different signaling pathways which, in turn, activate or not cell responses as previously shown for NADPH oxidase (26). Thus, CR3 appears to be a particularly elaborated receptor, able to activate different signaling pathways which lead to either type I or type II phagocytosis depending on the ligand that interacts with its multiple binding domains.

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References
