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Nonopsonic Phagocytosis of *Mycobacterium kansasii* by Human Neutrophils Depends on Cholesterol and Is Mediated by CR3 Associated with Glycosylphosphatidylinositol-Anchored Proteins

Pascale Peyron, Christine Bordier, Elsa-Noah N’Diaye, and Isabelle Maridonneau-Parini

Receptors involved in the phagocytosis of microorganisms under nonopsonic conditions have been little studied in neutrophils. Complement receptor type 3 (CR3) is a pattern recognition receptor able to internalize zymosan and C3bi-coated particles. We report that Abs directed against CR3 strongly inhibited nonopsonic phagocytosis of *Mycobacterium kansasii* in human neutrophils. In these cells CR3 has been found associated with several GPI-anchored proteins localized in cholesterol-rich microdomains (rafts) of the plasma membrane. Cholesterol sequestration by nystatin, filipin, or β-cyclodextrin as well as treatment of neutrophils with phosphatidylinositol phospholipase C to remove GPI-anchored proteins from the cell surface markedly inhibited phagocytosis of *M. kansasii*, without affecting phagocytosis of zymosan or serum-opsonized *M. kansasii*. Abs directed against several GPI-anchored proteins inhibited phagocytosis of *M. kansasii*, but not of zymosan. N-acetyl-D-glucosamine, which is known to disrupt interactions between CR3 and GPI proteins, also strongly diminished phagocytosis of these mycobacteria. In conclusion, phagocytosis of *M. kansasii* involved CR3, GPI-anchored receptors, and cholesterol. In contrast, phagocytosis of zymosan or opsonized particles involved CR3, but not cholesterol or GPI proteins. We propose that CR3, when associated with a GPI protein, relocates in cholesterol-rich domains where *M. kansasii* are internalized. When CR3 is not associated with a GPI protein, it remains outside of these domains and mediates phagocytosis of zymosan and opsonized particles, but not of *M. kansasii*. The Journal of Immunology, 2000, 165: 5186–5191.

Neutrophils are the first line of host defense against invading microorganisms. They exert their bactericidal activity by three mechanisms that generally lead to the destruction of microorganisms: recognition and phagocytosis of the infectious agent, generation of toxic oxygen derivatives through the activation of NADPH oxidase, and release of microbialicidal proteins from their specialized lysosomes (called azurophil granules) and granules.

The contact between neutrophils and micro-organisms occurs frequently in tissues after a lesion or in organs directly exposed to the environment, such as the respiratory tract or the digestive mucosa. Under these conditions, neutrophils are in contact with infectious agents that are not covered by serum opsonins, such as Abs and complement factors. Paradoxically, receptors able to recognize and internalize bacteria under nonopsonic conditions have been little studied in neutrophils, whereas opsonic receptors have been the aim of extensive studies. In macrophages, several pattern recognition receptors that directly recognize molecular determinants present at the surface of microbes have been described, but few of them are expressed in neutrophils (1–7).

The complement receptor 3 (CR3)\(^1\) has been described to function as a nonopsonic receptor in macrophages (3). It is also expressed in neutrophils (8). CR3 is a pattern recognition receptor initially identified as a specific receptor for the complement fragment C3bi. It is also a receptor for extracellular matrix proteins such as fibronectin and therefore belongs to the β2 integrin family, and finally, it is a lectin recognizing a large variety of sugars (3, 8, 9). CR3 is a heterodimer consisting of CD18, the protein common to β2 integrin family and CD11b, which contains the lectin site, the C3bi binding site, and the recognition domain of matrix proteins. This receptor has been involved in the selective internalization of pathogenic mycobacteria such as *Mycobacterium kansasii* in macrophages and in Chinese hamster ovary (CHO) cells stably expressing human CR3 (10, 11). *M. kansasii* remains a significant cause of human disease and is one of the most frequent nontuberculous mycobacterial pathogens isolated from clinical specimens (12). We have previously been involved in the study of bactericidal responses elicited by mycobacteria when they infect human neutrophils under nonopsonic conditions. We have reported that their phagocytosis elicits the production of O\(_2^-\) and the release of specific granule proteins, but the fusion of lysosomes with phagosomes was not triggered (13). Here we have tried to identify receptors involved in the phagocytosis of *M. kansasii* by human neutrophils. We report that CR3 plays a critical role in association with GPI-anchored proteins and depends on cholesterol.

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\(^3\) Abbreviations used in this paper: CR3, complement receptor 3; NADG, N-acetyl-D-glucosamine; Z, zymosan A; PIPLC, phosphatidylinositol phospholipase C; OZ, opsonised zymosan; CHO, Chinese hamster ovary.
Materials and Methods

Chemicals and Abs

Ficoll and dextran T500 were obtained from Amersham Pharmacia Biotech (Courtaboeuf, France). FITC was purchased from Molecular Probes (Eugene, OR). PBS, MEM, and HEPES were obtained from Life Technologies (Cergy-Pontoise, France). Nystatin, β-cyclodextrin, filipin, N-acetyl-D-glucosamine (NADG), zymosan A (Z), rabbit anti-mouse IgG-TRITC, goat anti-rabbit IgG-FITC, and goat anti-rabbit-TRITC were obtained from Sigma (Saint Quentin Fallavier, France). Mouse mAbs anti-CD55 (clone JS11, IgG1; Sixth International Workshop on Human Leucocyte Differentiation Ags) were provided by A. Bensussan (Institut National de la Santé et de la Recherche Médicale, Unité 448, Creteil, France). Mouse monoclonal anti-CD16b (clone 3G8, IgG1), 3G8-FITC, and rabbit polyclonal IgG anti-Hck were obtained from Sigma (Saint Quentin Fallavier, France). F(ab')2 of 3G8 were provided by C. Sautès (Institut National de la Santé et de la Recherche Médicale, Unité 225, Paris, France). Mouse monoclonal anti-CD14 (clone MY4, IgG2a K) and anti-CD66b (clone 80H3, IgG1 α) were purchased from Beckman Coulter (Gagny, France). Mouse anti-human CR3 (clone 2LPM19C, IgG K) and human anti-HLA class I (clone W6/32, IgG2a K) were obtained from Dako (Trappes, France). Mouse monoclonal anti-CD16b (clone 7.5.4, IgG1) was provided by J. L. Teillaud (Institut National de la Santé et de la Recherche Médicale, Unité 225, Paris, France). The rabbit Ab anti-mycobacterium, Camelia, has been previously described (10). Phosphatidylinositol phospholipase C (PIPLC) was purchased from Roche (Meylan, France).

Human neutrophils

Neutrophils were isolated from the blood of healthy donors by the dextran-Ficoll method as previously described (13), resuspended in MEM and 20 mM HEPES, pH 7.4, and maintained for 20 min at 37°C before stimulation.

Mycobacteria

M. kansasii (no. 124478, American Type Culture Collection, Manassas, VA) were prepared as previously described (13). Briefly, they were grown at 37°C as surface pellets in 250-ml flasks containing 100 ml of Sauton broth medium. The medium was discarded, and the pellets were disrupted by gentle shaking with glass beads (4 mm in diameter) for 30 min and resuspended in PBS, pH 7.4. To remove clumps, the bacterial suspensions were sedimented for 10 min, and supernatants were collected and centrifuged at 200 × g for 10 min. Mycobacteria in the supernatants were collected and counted under a microscope (Leica, Rueil-Malmaison, France) in a Thoma chamber, supplemented with 10% glycerol and stored at −80°C until use.

FITC staining of bacteria

Mycobacteria were labeled with FITC as previously described (13). Briefly, 1 × 10⁸ bacteria were added to 10 ml of 0.005% FITC in 0.2 M Na₂CO₃/NaHCO₃ and 150 mM NaCl buffer, pH 9.2, for 15 min. The bacteria were washed twice and resuspended in 1.5 ml of PBS, pH 7.4.

Opsonisation of FITC-stained bacteria or Z

Opsonisation was performed as previously described (13). Briefly, FITC-stained bacteria or Z (wall particles from M. kansasii) were incubated with FITC-stained bacteria (50 bacteria/cells) or with Z or OZ (20 particles/cells) for 20 min at 4°C and washed with cold MEM-HEPES to remove nonadherent particles. Internalization of particles was induced by placing the cells at 37°C for 45 min.

Phagocytosis measurement and indirect immunofluorescence

Measurement of phagocytosis was performed as previously described (10, 13). Neutrophils adhering on glass coverslips (15) were exposed to FITC-stained mycobacteria (50 bacteria/cells) for 45 min, washed three times, and fixed with 3.7% paraformaldehyde in PBS containing 15 mM sucrose, pH 7.4, for 30 min at room temperature. After neutralization with 50 mM NH₄Cl, slides were washed in PBS, and labeling of extracellular mycobacteria was performed using the rabbit polyclonal Ab, Camelia, directed against mycobacteria (1/50) revealed by tetramethylrhodamine isothiocyanate-conjugated second Abs (1/100). Fluorescent green and red bacteria were extracellular. Cells containing fluorescent green mycobacteria were counted by fluorescence microscopy (Leica).

Phagosomes of neutrophils that had ingested Z or opsonised zymosan (OZ) were visualized using anti-Hck Abs. This protein tyrosine kinase has been previously shown to accumulate at the membrane of phagocytes containing these particles (4, 16), facilitating the detection of Z- or OZ-positive phagocytes. Cells stimulated with Z or OZ were washed three times, fixed, and permeabilized in methanol at −20°C for 6 min. After two washes in PBS-0.1% Tween 20, cells were incubated with affinity-purified anti-Hck Abs (1/200) and then with FITC-conjugated secondary Abs to stain the membrane of phagosomes. Cells with Hck-stained phagosomes were counted by fluorescence microscopy. For each condition at least 100 cells were counted.

Drug treatment

Adherent neutrophils (7 × 10⁶ cells/ml in 20 mM MEM-HEPES, pH 7.4) were exposed to NADG (0.1 and 0.15 M), filipin (2 μg/ml), cyclodextrin (10 mM), or nystatin (25 μg/ml) for 15 min at 37°C. Cells were then incubated with FITC-stained bacteria (50 bacteria/cells) or with Z or OZ (20 particles/cells) as described above.

PIPLC treatment

Neutrophils in suspension were incubated with PIPLC (0.1 U/ml) for 30 min at 37°C. Cell adhesion on glass coverslips was then performed in the presence of PIPLC. To avoid translocation of the intracellular pools of GPI-linked receptors to the plasma membrane (17–19), cells were maintained at 4°C during the adhesion procedure and washed with cold MEM-HEPES (20 mM), pH 7.4. Cells were then exposed to mycobacteria (50 bacteria/cells) or OZ (20 particles/cells) for 20 min at 4°C and washed with cold MEM-HEPES to remove nonadherent particles. Internalization of particles was induced by placing the cells at 37°C for 45 min.

Ab treatment

Adherent neutrophils were incubated for 15 min at 37°C with Abs directed against GPI-anchored proteins of neutrophils (3G8, 2.5 μg/ml; 3G8 F(ab')₂, 2.5 μg/ml; 7.5-4, 2.5 μg/ml; MY4, 7.5 μg/ml; 80H3, 5 μg/ml; JS11, dilution 1/2), against a plasma membrane protein (anti-HLA class I, clone W6/32, 2.5 μg/ml) or against CR3 (2LPM19C, 2 μg/ml) and exposed to FITC-stained bacteria for 30 min at 37°C or to opsonised or nonopsonised Z for 15 min at 37°C. Cells were washed three times in PBS, pH 7.4, and fixed in paraformaldehyde.

FACS analysis

Cells in suspension were incubated with mouse anti-HLA (2.5 μg/ml) or FITC-conjugated anti-CD16b (3G8, 1/100) Abs at 4°C for 30 min and were washed twice in ice-cold PBS, pH 7.4, and fixed in paraformaldehyde. Anti-HLA Abs fixed on neutrophils were revealed by anti-mouse FITC-conjugated Abs (1/100). Fluorescence was measured by FACS analysis (FACScan, Becton Dickinson, Le Pont de Clai, France).

Results

CR3 is involved in the phagocytosis of M. kansasii by human neutrophils

To test the involvement of CR3 in phagocytosis, neutrophils were incubated with M. kansasii, Z, or OZ in the presence or the absence of the mouse mAbs 2LPM. Z and OZ are known to be internalized by CR3 (20). 2LPM Abs recognize an epitope located in the I domain of CD11b (11). We found that in the presence of Abs, the phagocytosis of Z and OZ was inhibited by 40 and 50%, respectively, whereas the phagocytosis of M. kansasii was inhibited by 90% (Fig. 1). CR3 has been shown to be physically and functionally associated with other receptors in the membranes of neutrophils, especially with GPI proteins (8, 21–24). Members of this family are attached to the plasma membrane via their carboxyl-terminal lipid modification, and most of them are constituents of membrane microdomains rich in cholesterol and glycosphingolipids, called rafts (25–27). Several reports suggest that the association of GPI-anchored receptors with CR3 enhances CR3 function (23, 28–30).

Cholesterol and GPI-linked proteins are involved in the phagocytosis of M. kansasii

To test the involvement of GPI-linked proteins in phagocytosis of M. kansasii, we first treated cells with cholesterol-scavenging (β-cyclodextrin) or -binding (filipin or nystatin) molecules, because it has been shown to affect the function of raft-associated proteins.
Neutrophils were exposed to 10 mM cyclodextrin for 15 min and mycobacteria, Z, or OZ were then added to the cells. As shown in Fig. 2A, phagocytosis of *M. kansasii* was strongly inhibited. Similar results were obtained with filipin and nystatin, indicating that despite their distinct modes of action on membrane cholesterol, the three molecules exert efficient inhibitory action on phagocytosis of *M. kansasii* (Fig. 2A). In addition, none of these molecules had a cytotoxic effect, because phagocytosis of Z was as efficient as that in control cells. Interestingly, when *M. kansasii* were serum opsonized, the inhibitory effect of cholesterol-depleting molecules was not observed (Fig. 2B), further supporting the lack of cytotoxicity discussed above and indicating that internalization of mycobacteria through opsonic receptors is not affected by cholesterol binding or scavenging molecules.

Because cholesterol depletion or sequestration could have affected the functions of GPI-anchored receptors (33, 35), we then determined whether the effect of removing GPI-linked proteins from the cell surface would have an effect on phagocytosis of *M. kansasii* comparable to that of cholesterol-chelating agents. Therefore, we treated cells with PIPLC to cleave the PI anchor and remove GPI receptors from the cell surface. Under our experimental conditions, PIPLC efficiently depleted the cell surface of a very abundant GPI protein in neutrophils, CD16b (FcγRIII, 200,000 sites/cell), as shown by FACS analysis (Fig. 3A). However, it has been previously described that not all the GPI-anchored proteins can be removed by PIPLC (36). After treatment of neutrophils with PIPLC, phagocytosis of *M. kansasii*, but not of Z, was inhibited (Fig. 3B). These results indicate that PIPLC has a specific inhibitory effect on phagocytosis of mycobacteria, most likely the result of removal of GPI-anchored proteins from the cell surface. It is interesting to note that internalization of OZ was not affected by removing CD16b from the cell surface, indicating that the other IgG receptors expressed in neutrophils in concert with complement receptors (37) are sufficient to maintain a constant rate of opsonic phagocytosis.

Several GPI-linked proteins are expressed in neutrophils. To identify the GPI-linked proteins involved in phagocytosis of *M. kansasii*, mouse mAbs directed against four GPI-linked proteins were tested. CD16b is a low affinity Fc receptor of IgG highly and specifically expressed in neutrophils that is associated with CR3 (24). CD14 is a receptor of LPSs complexed to the LPS-binding protein that reversibly associates with CR3 (24). To our knowledge, the physical association of CD55 (decay-accelerating factor) or CD66b with CR3 has not been examined, but CD66b has been involved in regulating the adhesive activity of CR3 (30). Dose-response curves were performed with Abs directed against these four GPI-linked proteins to determine the most efficient inhibitory concentration for each of them that was used in Fig. 4. All the Abs were found to inhibit phagocytosis of *M. kansasii*, but not phagocytosis of OZ (Fig. 4A) or Z (data not shown).

We checked that the effect of Abs was not the result of nonspecific binding of Abs to the cell surface of neutrophils. Similar experiments were performed with the anti-HLA class I Abs used. These Abs efficiently bound to the cell surface, as shown by FACS analysis (Fig. 4B) but did not interfere with phagocytosis of OZ, *M. kansasii* (Fig. 4A), or Z (not shown). Moreover, F(ab’)_2 of 3G8 were as efficient as the corresponding Ig to inhibit phagocytosis of *M. kansasii* (Fig. 4A).

In conclusion, phagocytosis of *M. kansasii* is inhibited by 1) molecules that sequester cholesterol; 2) PIPLC, which removes...
GPI-anchored proteins from the cell surface; and 3) Abs directed against CR3 or GPI receptors. These data suggest that GPI receptors associate with CR3 in cholesterol-rich domains, triggering the entry of these bacteria in human neutrophils.

3-NADG inhibits phagocytosis of mycobacteria

To determine whether association of GPI-linked proteins to CR3 is critical for phagocytosis of *M. kansasii*, neutrophils were incubated with NADG, a saccharide previously shown to disrupt the interaction between CR3 and GPI-linked receptors such as CD16b and CD59 (38, 39). This saccharide binds to the lectin site of CR3 shown to be involved in the recognition of GPI-linked proteins of Z, but not of mycobacteria (10, 40). As expected, in the presence of NADG, phagocytosis of Z, but not that of OZ, was inhibited. Phagocytosis was measured, and the values are the mean ± SEM of three separate experiments performed in duplicate. The percentages of cells having ingested *M. kansasii*, Z, and OZ were 27 ± 8, 45 ± 10, or 70 ± 3%, respectively.

Discussion

In this paper we show that phagocytosis of *M. kansasii* by human neutrophils is specifically inhibited by cholesterol-sequestering or -chelating reagents that did not affect the phagocytosis of Z or OZ. While this manuscript was being reviewed, similar cholesterol requirement for the phagocytosis of *M. tuberculosis* and *M. bovis* Calmette-Guérin bacillus by murine macrophages was reported (41). Phagocytosis of serum-opsonized mycobacteria was not inhibited, indicating that opsonic receptors are not cholesterol dependent. Moreover, phagocytosis of *M. kansasii*, but not that of Z or OZ, was inhibited by treatment of the cells with PIPLC, which removes part of the GPI-linked proteins from the cell surface. Because this type of proteins is present in domains on the cell surface that are rich in cholesterol, known as rafts (25–27), and because the function of these proteins is altered by cholesterol depletion (33, 35), our results suggest that the entry of nonopsonized *M. kansasii* into neutrophils takes place at rafts.

The nonopsonic receptors involved in phagocytosis of *M. kansasii* in neutrophils are not known. We found that Abs against CR3 inhibit the entry of *M. kansasii*, implying that CR3 is one such receptor. CR3 is known to associate with several GPI-anchored receptors (8, 21–24), suggesting that CR3 may be localized in rafts. However, we also found that CR3 is involved to some extent in the phagocytosis of Z and OZ, whose phagocytosis was not raft-dependent. Moreover, phagocytosis of *M. kansasii*, but not that of Z or OZ, was inhibited by treatment of the cells with PIPLC, which removes part of the GPI-linked proteins from the cell surface. Because this type of proteins is present in domains on the cell surface that are rich in cholesterol, known as rafts (25–27), and because the function of these proteins is altered by cholesterol depletion (33, 35), our results suggest that the entry of nonopsonized *M. kansasii* into neutrophils takes place at rafts.

Binding of GPI-linked proteins by CR3 could result in its translocation to rafts, and if we assume that these events would render CR3 outside of rafts. Therefore, there must also be a population of CR3 outside of rafts.

FIGURE 3. PIPLC inhibits phagocytosis of *M. kansasii*. Human neutrophils were incubated with 0.1 IU/ml PIPLC for 30 min. A, Surface expression of the GPI-anchored protein CD16b was analyzed by flow cytometry. Gray line, neutrophils; dark gray, CD16b-FITC stained control neutrophils; black, CD16b-FITC stained neutrophils treated by PIPLC. One experiment representative of three is shown. The fluorescence is expressed in arbitrary units and is measured on a complete cell population. B, Z, OZ, or *M. kansasii* was added to neutrophils treated, or not, with PIPLC. Phagocytosis was measured, and the values are the mean ± SEM of three separate experiments performed in duplicate. The percentages of cells having ingested *M. kansasii*, Z, and OZ were 27 ± 8, 45 ± 10, or 70 ± 3%, respectively.

FIGURE 4. Abs directed against GPI proteins inhibit phagocytosis of *M. kansasii* by human neutrophils. A, Human neutrophils were incubated for 15 min with Abs directed against GPI proteins (anti-CD66b, 80H3; anti-CD55, JS11; anti-CD16b, 7.5.4 and 3G8; anti-CD14, MY4), with Fab′(a′)2 from 3G8) or anti-HLA class I, and OZ or *M. kansasii* was added. Phagocytosis was measured, and the values are the mean ± SEM of three separate experiments performed in duplicate. The percentages of cells having ingested *M. kansasii* and OZ were 35 ± 8 and 68 ± 7%, respectively. B, Association of anti-HLA Abs with neutrophils was analyzed by flow cytometry. Neutrophils were treated with anti-HLA Abs, fixed, and stained with FITC-coupled rabbit anti-mouse Abs. Gray line, no treatment (neutrophil autofluorescence); black gray, neutrophils treated with anti-HLA and FITC-secondary Abs; black, anti-HLA Abs omitted. The fluorescence is expressed in arbitrary units and is measured on a complete cell population. One experiment representative of two is shown.
CR3, which, consequently, remains unable to internalize protein could be its receptor. More likely, binding of Abs to GPI-anchored proteins in CHO cells. In contrast, when CR3 is not associated with cholesterol and association with GPI-anchored proteins in CHO cells, CR3 needs to associate with GPI receptors in cholesterol-rich domains. They cooperate with CR3 to mediate intracellular signaling, as demonstrated by several groups (22–24). Conversely, association of GPI-linked proteins with CR3 has been shown to enhance the activity of CR3 (23, 29, 30), probably because this association produces conformational changes in CR3. Therefore, to internalize M. kansasii in human neutrophils, we propose that CR3 needs to associate with GPI receptors in cholesterol-rich domains. In contrast, when CR3 is not associated with a GPI protein, it remains outside of cholesterol-rich domains where it can mediate phagocytosis of Z and OZ, but not that of M. kansasii.

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References


FIGURE 5. NADG inhibits phagocytosis of M. kansasii by human neutrophils. Neutrophils were incubated with NADG at different concentrations (0.1 and 0.15 M) for 15 min, and Z, OZ, or M. kansasii was added. Phagocytosis was measured, and the values are the mean ± SEM of five separate experiments performed in duplicate. The percentages of cells having ingested M. kansasii, Z, and OZ were 31 ± 4, 38 ± 6, and 70 ± 1%, respectively.

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