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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.


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Materials and Methods

Chemicals and Abs

Ficoll and dextran T500 were obtained from Amersham Pharmacia Biotec (Courtabœuf, 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 (NAGD), 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 Leukocyte Differentiation Antigens) 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 Santa Cruz Biotechnology (Le Perray en Yvelines, France). F(ab')2 of 3G8 were provided by C. Sautes (Institut National de la Santé et de la Recherche Médicale, Unité 225, Paris, France). Mouse monoclonal anti-CD14 (clone MY4, IgG2a) and anti-CD66b (clone 80H3, IgG1) were purchased from Beckman Coulter (Gagny, France). Mouse anti-human CR3 (clone 2LPM19C, IgG1 K) and human anti-HLA class I (clone W6/32, IgG2a) 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) (14). 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 pellicles 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 × 10⁹ 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, has been previously described (10). Phosphatidylinositol phospholipase C (PIPLC) was purchased from Roche (Meylan, France).

Drug treatment

Adherent neutrophils (7 × 10⁷ cells/ml in 20 mM MEM-HEPEs, pH 7.4) were exposed to NADG (0.1 and 0.15 M), filipin (2 μg/ml), 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 μM) 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; 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 nonopsonized 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 Claix, 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). Therefore, we examined whether GPI proteins could be part of the phagocytic machinery for mycobacteria.

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.
A shown in Fig. 2, phagocytosis of min and mycobacteria, Z, or OZ were then added to the cells. As (31–34). Neutrophils were exposed to 10 mM cyclodextrin for 15 min and 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, 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.

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 affected by cholesterol-depleting or -sequestering agents. Therefore, there must also be a population of CR3 outside of 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 capable of binding M. kansasii, this would explain why we could equally well inhibit M. kansasii phagocytosis by Abs against a variety of GPI-linked proteins. Therefore, we think that CR3, rather than any specific GPI-linked proteins, functions as the M.
CR3, which, consequently, remains unable to internalize proteins might impair (steric hindrance) their association with protein could be its receptor. More likely, binding of Abs to GPI is necessary to mediate phagocytosis. If the human CR3 is expressed in neutrophils are heavily glycosylated (45). This interaction between CD16b or CD87 and CR3 is mediated by the glycosylated or associated with proteins or sugars (3, 8, 44). The interactions of the sugar specificity and molecular location of the β-glucan binding lectin site of complement receptor 3 (CD11b/CD18) (1). J. Immunol. 156:1253.


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