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Superoxide Anion Generation in Human Milk Macrophages: Opsonin-Dependent *Versus* Opsonin-Independent Stimulation Compared with Blood Monocytes

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ABSTRACT

Macrophages are believed to play an important role within the immunoprotective effects of human breast milk. It was the purpose of this study to evaluate the capability of human milk macrophages (MMΦ) to generate superoxide anions (O_2^-) in comparison with peripheral blood monocytes (BMo) after stimulation with opsonized and unopsonized zymosan. Potential inhibitors of attachment and phagocytosis such as mannose and cytochalasin B were used. Expression of the mannose receptor on MMΦ was demonstrated by staining with MAb. BMo generated more O_2^- than MMΦ (417 ± 79 *versus* 216 ± 15 nmol O_2^-/mg protein, $p < 0.05$) after stimulation with opsonized zymosan. When unopsonized zymosan was used as a serum-independent stimulus, BMo generated slightly less O_2^- in comparison with MMΦ (150 ± 34 *versus* 176 ± 18 nmol O_2^-/mg protein, $p < 0.05$). These findings imply a higher proportion of opsonin-independent phagocytosis in MMΦ than in BMo (82 *versus* 36%). Preincubation with mannose resulted in a significantly higher reduction of O_2^- generation in MMΦ compared with BMo stimulated with opsonized zymosan, whereas no difference was found when unopsonized zymosan was used. After addition of cytochalasin B, equal inhibition of O_2^- generation was observed regardless of the cell type or stimulus used. Thus, MMΦ are stimulated to a greater extent by serum-independent mechanisms than BMo. As opsonins like complement or IgG are rare in the colostrum and the neonatal intestinal environment, such a differentiation toward serum-independent phagocytic abilities could play an important role for protective functions of human MMΦ. Possible involvement of the mannose receptor and the β-glucan receptor in this specialization are discussed. **(*Pediatr Res* 49: 435–439, 2001)**

Abbreviations

MMΦ, milk macrophages
BMo, blood monocytes
nmol O_2^-·mg p^{-1}·$30'^{-1}$, nmol O_2^-·mg protein^{-1}·30 min^{-1}
MR, mannose receptor
CR3, complement receptor type 3

Besides well-known nutritional benefits, transfer of antimicrobial activity is of great importance in infant breast-feeding. A great variety of humoral defense factors (*e.g.* secretory Ig, especially sIgA, lactoferrin, lysozyme, oligosaccharides, mucins, and others) contribute to the beneficial effects. In addition, large numbers of viable cells are present in human colostrum and breast milk with a high proportion of macrophages likely being responsible for antiinfectious properties.

These human MMΦ with a diameter of 18 to 40 μm contain a high amount of phagocytosed lipids, "foamy cells." Their morphologic and cytochemical properties are similar to differentiated macrophages. For example, they bear Fc receptors for different subclasses of IgG, IgA, and C3b receptors and synthesize various humoral defensive factors such as complement factors, lactoferrin, and lysozyme (1–5).

Because human MMΦ remain viable in conditions similar to those in the small intestine (6), show relative resistance to an environment with a pH <3, and resist trypsinization (7), it seems very likely that MMΦ can develop their immunoprotective functions within the gastrointestinal tract of the breast-fed baby.

Interaction of macrophage membrane receptors with complementary coating substances on a pathogen's surface is well described as opsonophagocytosis. These coatings (opsonins), derived from various sites of the hosts immune system, consist of bridging serum factors such as Ig, the iC3b fragment of C3 complement factor, C-reactive protein, surfactant proteins A and D, and the mannose-binding protein (8).

Interaction of carbohydrate-binding proteins termed lectins with complementary carbohydrate chains is another primary mechanism of mediating attachment of pathogens. This phago-

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cytic process, known as serum independent or lectinophagocytosis, is promoted, for example, through the MR or a lectin site on the CD11b/CD18 (MAC-1) integrin, the β-glucan receptor (8).

After recognition and attachment of pathogenetic particles, the process of phagocytosis progresses with different patterns of internalization and mostly culminates in the triggering of the respiratory burst with the release of microbicidal oxygen metabolites. It is hypothesized that due to a lower availability of opsonizing factors in the milk (9) and the gastrointestinal tract of the infant, human MMΦ are activated by other mechanisms than BMo.

Therefore, this study was undertaken to investigate opsono- and lectinophagocytic properties of human MMΦ by measuring the superoxide anion (O_2^-) production in comparison with human BMo after stimulation with opsonized and unopsonized zymosan particles. D-Mannose and cytochalasin B as potential inhibitors were used to evaluate attachment and engulfment mechanisms.

METHODS

Collection, Preparation, and Culture of Cells

Human milk was collected from 38 healthy lactating women 1–6 d postpartum by hand expression with informed consent at the Department of Gynecology and Obstetrics, Heinrich-Heine-Universität Düsseldorf, Germany, after having obtained approval of the Clinic's Review Board. The samples were generally obtained before the infants were fed and stored at room temperature for up to 4 h in sterile containers.

The milk was skimmed by 1:1 dilution with PBS and centrifuged at 4°C and 600 × *g* for 10 min. The pellet was resuspended in 25 mL PBS, layered on 25 mL Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and centrifuged at 20°C and 1000 × *g* for 20 min. The mononuclear interface cell layer was washed in PBS and RPMI (GIBCO, NY, U.S.A.) (supplemented with penicillin, gentamicin, HEPES, FCS, and glutamine) and resuspended in RPMI (with supplements). Purity (>90%) of the cell preparation was assessed by naphthyl acetate esterase staining. Cells were enumerated by cell counter (Coulter, Krefeld, Germany) and adjusted at a concentration of 2.5×10^5 cells/mL. Monocytic viability of ≥95% was assured by trypan blue exclusion. Two milliliters of the cell suspension were prepared in plastic culture dishes, and cells were allowed to adhere for 2 h at 37°C and 5% CO_2, then washed vigorously with PBS to remove nonadherent cells.

Monocytes were isolated from heparinized human blood from 64 healthy volunteers as described above by Ficoll-Hypaque gradient and then treated like the MMΦ.

The protein content in the samples was determined according to the method of Lowry *et al*. (10). Only samples with a protein content between 40 and 100 μg were used (11). The cells were stored in PBS on ice (maximum, 15 min) until use.

O_2^- Production

O_2^- production was measured by reduction of superoxide dismutase-sensitive cytochrome *c*. Addition of 2 mL *N*-ethylmaleimide stopped the induced oxidative metabolism after treatment with the stimulants for 30 min. Reduction of cytochrome *c* was quantified spectrophotometrically (550 nm). The assay was repeated with superoxide dismutase to correct for oxygen-independent reduction of cytochrome *c*. Results were expressed in nmol O_2^-/mg protein.

Stimulating Agents

Opsonized and unopsonized zymosan (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a stimulus. Serum from healthy donors was taken for opsonization. The zymosan particles were autoclaved for 30 min, washed twice with PBS, and resuspended in PBS. The samples were adjusted to 2×10^5 particles/μL and stored at −70°C until further use.

Inhibitors

Mannose and cytochalasin B (Sigma Chemical Co., St. Louis, U.S.A.) were used as potential inhibitors. Two milliliters of each substance was incubated with the isolated cells for 15 min at 37°C before the stimulation assay. Mannose was prepared in 0.1, 0.25, and 0.5-M solutions, and cytochalasin B was used at a concentration of 1 μg/mL. All assays were performed in duplicate.

Expression of MR

Phagocytes were stained with a monoclonal murine antibody generated against the human MR (Pharmingen, San Diego, CA, U.S.A.). BMo and MMΦ were prepared as described above and subsequently incubated with the anti-MR antibodies (20 μL per pellet) for 10 min, washed with PBS, and centrifuged at 300 × *g* for 10 min at 20°C. Staining was evaluated using a fluorescence microscope (Zeiss Optics, Göttingen, Germany).

Statistical Analysis

Statistical analysis was performed by using the paired *t* test. An α error of $p < 0.05$ was regarded as significant.

RESULTS

Stimulation without Inhibitors

Without any additional stimuli, a O_2^- production of 40 ± 3 nmol O_2^-·mg p^{-1}·$30'^{-1}$ in MMΦ and 47 ± 4.7 nmol O_2^-·mg p^{-1}·$30'^{-1}$ in BMo was measured. After stimulation with opsonized zymosan, BMo generated 417 ± 79 nmol O_2^-·mg p^{-1}·$30'^{-1}$, and MMΦ generated 216 ± 15 nmol O_2^-·mg p^{-1}·$30'^{-1}$. When unopsonized zymosan was used, BMo produced 150 ± 35 nmol O_2^-·mg p^{-1}·$30'^{-1}$, and MMΦ generated 176 ± 18 nmol O_2^-·mg p^{-1}·$30'^{-1}$. Thus, peripheral BMo released a higher absolute amount of O_2^- than human MMΦ when stimulated with opsonized zymosan, whereas O_2^- production after activation with unopsonized zymosan demonstrated approximately equal release of the measured oxygen metabolites. Notably, MMΦ showed a significantly higher proportion of O_2^- generation under opsonin-independent stimulation (100 to 82%) than

BMo (100 to 36%) in relation to opsonin-dependent stimulation (Fig. 1).

Stimulation after Preincubation with Mannose

After addition of D-mannose before stimulation with opsonized zymosan, BMo reacted with a significant reduction in O_2^- generation (302 ± 62, 247 ± 58, and 162 ± 53 nmol O_2^-·mg p^{-1}·$30'^{-1}$ for 0.1, 0.25, and 0.5 M mannose, respectively; 0.5 M mannose: 61%, $p < 0.0025$). MMΦ showed lower O_2^- production (139 ± 18, 84 ± 14, and 39 ± 1 nmol O_2^-·mg p^{-1}·$30'^{-1}$ after preincubation with the above-mentioned concentrations of D-mannose, respectively; 0.5 M mannose: 82%, $p < 0.0005$). These results suggest that MMΦ can be inhibited to a greater extent by mannose than BMo under conditions of serum opsonization (Fig. 2).

Preincubation of D-mannose after stimulation with unopsonized zymosan revealed a decrease in O_2^- output in BMo (89 ± 15, 63 ± 11, and 30 ± 5 nmol O_2^-·mg p^{-1}·$30'^{-1}$ for 0.1, 0.25, and 0.5 M mannose, respectively; 0.5 M mannose: 80%, $p < 0.05$) and a reduction in MMΦ (99 ± 13, 58 ± 7, and 32 ± 5 nmol O_2^-·mg p^{-1}·$30'^{-1}$; 0.5 M mannose: 82%, $p < 0.0005$). Thus, no significant difference could be found between the two cell populations when unopsonized zymosan was used (Fig. 3).

Stimulation after Preincubation with Cytochalasin B

After the cells were treated with cytochalasin B (1 μg/mL), a significant reduction of O_2^- generation was detected. When they were stimulated with opsonized zymosan, we found that BMo showed a decrease from 355 ± 27 to 264 ± 32 nmol O_2^-·mg p^{-1}·$30'^{-1}$ ($p < 0.0005$), whereas MMΦ exhibited a decrease from 214 ± 24 to 131 ± 14 nmol O_2^-·mg p^{-1}·$30'^{-1}$ ($p < 0.005$). When unopsonized zymosan was used as a stimulant, a decrease from 150 ± 14 to 111 ± 13 nmol O_2^-·mg p^{-1}·$30'^{-1}$ was detected in the BMo population ($p < 0.0005$), whereas MMΦ displayed a reduction from 205 ± 6 to 125 ± 21 nmol O_2^-·mg p^{-1}·$30'^{-1}$ ($p < 0.025$). We found no significant difference in the inhibition of O_2^- release induced by cytochalasin B regardless of the cell type or stimulus used (Fig. 4).

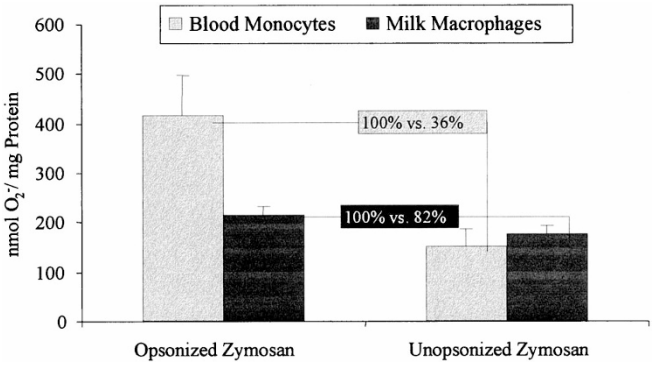

Figure 1. O_2^- production (nmol O_2^-·mg p^{-1}·$30'^{-1}$) of BMo and MMΦ after incubation with opsonized and unopsonized zymosan without addition of inhibitors. BMo showed an almost equal generation of O_2^- to MMΦ after stimulation with unopsonized zymosan, whereas release of O_2^- was markedly higher when stimulated with opsonized zymosan. When O_2^- production was compared in each cell subpopulation, the proportion of serum-independent stimulation was significantly higher in MMΦ (82% in MMΦ, 36% in BMo compared with challenge with opsonized zymosan).

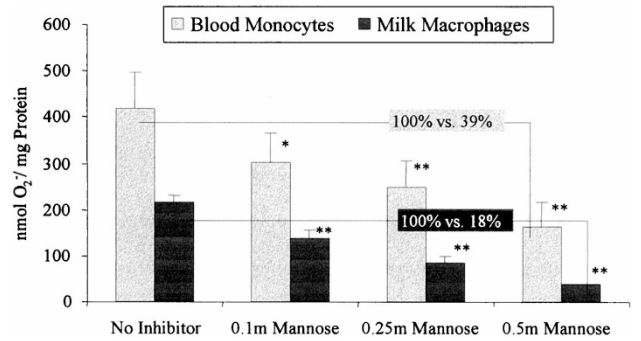

Figure 2. O_2^- production (nmol O_2^-·mg p^{-1}·$30'^{-1}$) of BMo and MMΦ after incubation with opsonized zymosan as a serum-dependent stimulus after addition of either 0.1, 0.25, or 0.5 M D-mannose. Treatment with increasing doses of mannose resulted in decreased production of O_2^- in both types of phagocytes. Inhibition of O_2^- output was significantly greater in MMΦ than in BMo compared with stimulation without mannose; when 0.5 M mannose was applied, a reduction of 82% was detected in MMΦ, whereas BMo reacted with a decrease of 61% in O_2^- generation (*$p < 0.05$, **$p < 0.005$).

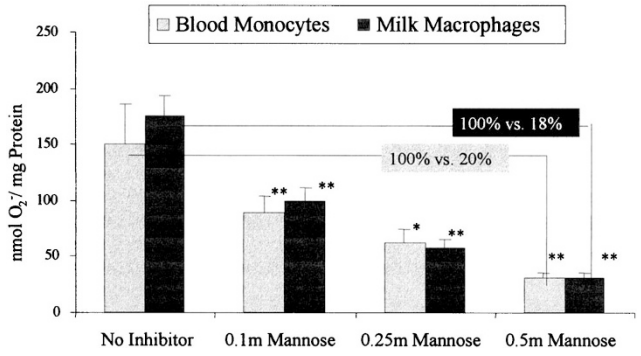

Figure 3. O_2^- production (nmol O_2^-·mg p^{-1}·$30'^{-1}$) of BMo and MMΦ after incubation with unopsonized zymosan as a serum-independent stimulus after addition of either 0.1, 0.25, or 0.5 M D-mannose. Both cell populations reacted with a comparable decrease in O_2^- output after pretreatment with D-mannose in relation to stimulation without inhibitor, 82% in MMΦ and 80% in BMo after preincubation with 0.5 M mannose (*$p < 0.05$, **$p < 0.005$).

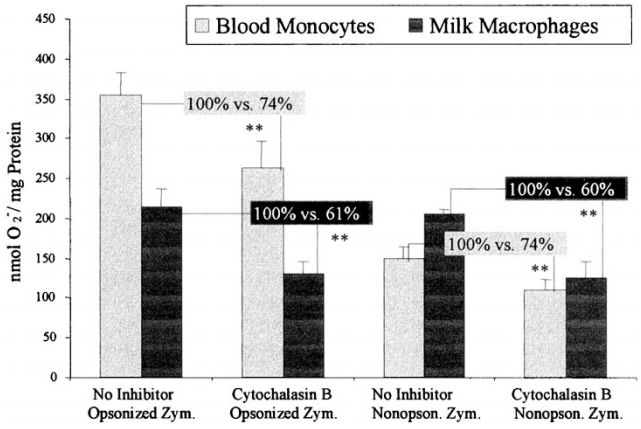

Figure 4. O_2^- production of BMo and MMΦ after incubation with opsonized and unopsonized zymosan after addition of cytochalasin B. Pretreatment with the cytoskeleton inhibitor resulted in comparable inhibition of O_2^- production in both BMo and MMΦ (**$p < 0.005$).

Detection of MR

Detection of the MR on MMΦ was performed by fluorescent staining using MAb directed against this recognition site. Only MMΦ showed an expression of the MR.

DISCUSSION

Stimulation without Inhibitors

Different from our findings in which we demonstrated a higher proportion of opsonin-independent stimulation in MMΦ, O_2^- production in human MMΦ has been reported to be equal to BMo after stimulation with phorbol myristate acetate or opsonized zymosan (7, 12, 13). These results, though, were related to the amount of cells in the sample (nmol O_2^-/5 × 10^5–10^6 cells). Because MMΦ are much bigger in diameter than monocytes and thus have a larger surface area, a greater number of relevant receptors per cell could account for a bias within these investigations, as O_2^- production in MMΦ may be elevated. Given that variability in the measurement of O_2^- production occurs in samples with a protein content below 35 μg protein (14), the O_2^- generation was related to the absolute amount of protein instead of the quantity of cells.

Different patterns of receptors on the surface of the phagocytes regarding their number or quality (*e.g.* Fc receptor and CR3 on one hand, MR and β-glucan receptor on the other) could be a possible indication for the propagated specialization of MMΦ. Reduced amount of opsonins (such as complement and Ig) in the colostrum (9) and the gastrointestinal tract of the neonate might lead to a relatively better serum-independent stimulation of MMΦ.

Stimulation after Preincubation with Mannose

Unopsonized zymosan. Zymosan as a derivative of the cell wall of *Saccharomyces cerevisiae* is composed of α-D-mannan and β-D-glucan, two carbohydrate polymers, with glucan being the most abundant component (15, 16). Binding and phagocytosis of unopsonized zymosan was reported to be dependent on the expression of the MR on human (17) and murine macrophages (18, 19). Other studies showed the dependency of O_2^- release in murine and rabbit macrophages on the MR (20, 21).

Besides difficulties in the direct transfer of research results derived from different species, some aspects of our study indicate that the MR may not play a major role; rather, another type of membrane receptor may be responsible for zymosan signaling and consecutive respiratory-burst activities in human BMo and MMΦ.

Monocytes do not express the MR until they are cultured for several days (22, 23). We confirmed this by demonstrating negative staining of BMo with FITC-labeled anti-human MR MAb within the cultivation periods of monocytes used in our experiments. MMΦ, in contrast, do bear MR on their membrane as determined in this study by staining with MAb.

In our assay, both types of cells produced approximately equal amounts of O_2^- when challenged with zymosan particles without any opsonizing serum factors. Addition of D-mannose in different concentrations resulted in inhibition of O_2^- release to the same extent in both types of cells. This same reaction to stimulation and inhibition by both types of phagocytes (one being devoid of the MR, one being equipped with it) render an involvement of this type of receptor unlikely in the production of O_2^-.

These findings are supported by a recent report of Astarie-Dequeker *et al*. (24) who showed that phagocytosis of unopsonized zymosan through the MR did not result in triggering of O_2^- production. These investigators could demonstrate that the uptake of zymosan particles by human monocyte-derived macrophages was dependent on the MR as well as on another membrane component, the β-glucan receptor, which is located on the complement receptor type 3 (CR3). Even though the internalization of unopsonized zymosan was also mediated by the MR, an observation that has been confirmed by Lombard *et al*. (25), the O_2^- generation itself was triggered only by phagocytosis via the β-glucan receptor.

This lectin-like β-glucan receptor has been located on the $\alpha_m\beta_2$ integrin CR3 (CD11b/CD18, Mac-1), which is composed of the α (CD11b) and β (CD18) subunits. The β-glucan site was found to be situated on the α chain C-terminal to the I-domain, distinct from the binding sites for iC3b, ICAM-1, fibrinogen, and clotting factor X (26, 27).

Our results (with stimulation by unopsonized zymosan and inhibition with d-mannose to the same extent in both types of cells) could be the consequence of a comparable distribution of the β-glucan receptor on BMo and MMΦ because the MR does not seem to be involved in the release of O_2^- anions. Considering the sugar specificity of this lectin site, the demonstrated concentration-dependent functional impairment of zymosan-induced O_2^- production could be explained by the inhibition of the β-glucan receptor by D-mannose. Supporting this, an investigation by Thornton *et al*. (28) revealed the lectin site of CR3 to have a broader specificity for certain polysaccharides than originally appreciated. In this regard, SZP, a soluble zymosan polysaccharide, which blocked the binding site to the same extent as various β-glucan preparations, was unexpectedly found to consist primarily of mannose.

Opsonized zymosan. A greater mannose-exerted inhibition of O_2^- production in MMΦ and a suggested similar expression of β-glucan/CR3 receptors in the two cell populations lead to the conclusion that a different type of opsonin receptor being disproportionally distributed accounts for the effect of this uneven stimulation and inhibition in connection with opsonized zymosan.

In this respect, a decreased expression of all three subclasses of Fc receptors (CD16, CD32, CD64) on MMΦ has been described by Rivas *et al*. (14). Participation of Ig in the opsonization of zymosan has been demonstrated in a study on respiratory burst in human granulocytes (29). Even zymosan-specific IgG antibodies have been identified to enhance alternative pathway activities in human serum (30).

Especially, complement factors such as C3b or iC3b are necessary in the opsonization of zymosan (31). A concerted interaction between CR3 and the β-glucan lectin site (with the complement receptor being the primary binding site and the β-glucan receptor being the function-triggering moiety) has been postulated in the synthesis of platelet-activating factor in human monocytes (32). Moreover, in human neutrophils, binding of complement-opsonized yeast is related to the binding

site for iC3b on CR3, whereas ingestion and respiratory burst depend on coupling with the β-glucan binding site (33).

Monocytes and MMΦ have been shown to secrete essential factors for activation and propagation of the alternative complement pathway (1, 34), so they are capable of local zymosan opsonization via autocrine liberation of complement factors (35, 36).

Taken together, two major opsonin-dependent receptors with different distributions, the CR3 receptor being expressed approximately to the same extent in both cell types and the Fc receptor being more abundant on the surface of BMo, may be responsible for the results found in our studies.

Stimulation after Preincubation with Cytochalasin B

In our experiments, both MMΦ and BMo reacted with an almost equal reduction in O_2^- generation after treatment with cytochalasin B when stimulated with either opsonized or unopsonized zymosan. These findings suggest comparable engulfment mechanisms of foreign particles in these phagocyte populations that seem to be dependent on an intact microfilamentous system.

Cytoskeletal integrity has been shown to be required for internalization of opsonized particles such as formation of phagosomes or lamellipodia (37). Cytochalasin B, as an agent to disrupt microfilaments by interfering with actin polymerization, was reported to inhibit endocytosis (38, 39), degradation of proteins by macrophages (40), and formation of foreign-body giant cells by macrophages (41), so an interference with the production of reactive oxygen metabolites seems possible as well.

In summary, we find that the macrophage population in human milk is capable of reacting with the release of O_2^- to both opsonized and unopsonized particles with a higher proportion of serum-independent phagocytosis. The higher amount of these "lectinophagocytic" responses possibly reflects the specialization of these phagocytes to the specific neonatal environment.

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