INFECTION AND IMMUNITY, Nov. 1997, p. 4747–4753 Vol. 65, No. 11
0019-9567/97/$04.00+0

Azurophilic Granules of Human Neutrophils Contain CD14

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Received 22 May 1997/Returned for modification 2 July 1997/Accepted 31 August 1997

CD14, the leukocyte receptor for lipopolysaccharide (LPS), is important in the response of human polymorphonuclear leukocytes (PMNs) to infection with gram-negative bacteria. The level of CD14 on the PMN surface increases after exposure to some inflammatory stimuli such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). These newly expressed CD14 molecules probably come from an intracellular pool of preformed receptors. We sought to further characterize PMN CD14 expression, upregulation, and shedding and to define the intracellular location of CD14 molecules. Our results demonstrate that both LPS and fMLP significantly increased CD14 cell surface expression; however, neither phorbol myristate acetate (PMA) or A23187 increased receptor levels on the PMN surface. Neither fMLP, PMA, or A23187 stimulated the release of soluble CD14 from PMNs. Intracellular CD14 was observed in >90% of PMNs examined by flow cytometry and confocal microscopy. Additional analyses using CD14 enzyme-linked immunosorbent assays and electron microscopy studies, examining PMN granules separated by discontinuous sucrose or Percoll gradients, showed that CD14 was present in both the plasma membrane-secretory vesicle fractions and azurophilic granules.

The interaction of lipopolysaccharide (LPS) with the immune system, a consequence of gram-negative infection, initiates the systemic inflammatory response syndrome, which can progress ultimately to multiple organ dysfunction syndrome. Polymorphonuclear leukocyte (PMN) activation, a key process in the initiation of systemic inflammatory response syndrome, begins with the interaction of LPS, in conjunction with LPS binding protein (LBP), with CD14 (34, 40, 46). CD14 is a leucine-rich, 55-kDa protein that is anchored to the plasma membrane by a glycosylphosphotidylinositol linkage (8, 21, 37). CD14 is present on the plasma membrane of >95% of monocytes and, for a decade, was only considered to be a marker for monocytic cells. Since then CD14 has been observed on basophils, B cells (27, 39) and 20 to 40% of PMNs (4, 24). CD14 also is found in the serum and urine of normal humans as a 50-kDa protein, which originates from the plasma membrane of leukocytes, following its release by a membrane-bound protease (5, 48). Shedding of leukocyte CD14 is constitutive and inducible. This soluble form of CD14 (sCD14) appears to have two main functions. First, sCD14 neutralizes the LPS-LBP complex, thereby preventing further stimulation of the inflammatory response (31, 35). Second, sCD14 also can insert into the plasma membrane of cells that do not normally express CD14, such as endothelial cells, enabling LPS activation of these cells (9).

The expression of CD14 on the cell surface can be affected by many inflammatory signals. PMN CD14 cell surface expression can be increased by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) tumor necrosis factor, or colony-stimulating factors (granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor) (45). This increase in cell surface receptor expression occurs within 30 min of stimulation and is not inhibited by cyclohexamide (43, 45). These observations indicate that preformed CD14 may be stored within an intracellular compartment (45). Although CD14 has only been observed on a low percentage of PMNs the CD14 transcript is present at high levels within PMNs (15). This would support the hypothesis that PMNs contain intracellular pools of CD14 that are available for mobilization to the cell surface. The localization of such pools has been addressed recently by Detmers et al., who showed that preformed CD14 is present within the secretory vesicles of PMNs (7).

We evaluated the location of intracellular CD14 by using multiple lines of investigation. Because LPS and fMLP preferentially induce the mobilization of secretory vesicles (36) and phorbol myristate acetate (PMA) and A23187 induce the mobilization of specific granules (36, 44), we tested the hypothesis that CD14 is present within secretory vesicles but not within specific granules of PMNs. Our results indicate that LPS and fMLP increase PMN CD14 receptor expression but that PMA and A23187 do not. However, neither fMLP, PMA, or A23187 induce the release of sCD14. CD14 is present within the cytoplasm of nearly all PMNs, as determined by flow cytometry and confocal microscopy. In addition to secretory vesicles, enzyme-linked immunosorbent assays (ELISA) and electron microscopy show that CD14 is present within the azurophilic granules of human PMNs.

MATERIALS AND METHODS

PMN and lymphocyte isolation and culture. All chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, Mo.), and all sterile noncharged polystyrene plasticware was obtained from Corning, Inc. (Corning, N.Y.). All culture media and solutions were made using LPS-free sterile water (Baxter, McGraw Park, Ill.) and were filtered with a Falcon 7105 sterile 0.22-μm-pore-size filter (Becton Dickinson, Oxnard, Calif.). PMNs, from healthy human volunteers, were separated from whole blood within 15 min of being drawn. PMN separation was accomplished by using a neutrophil isolation medium (NIM) centrifuge gradient (containing Ficoll-Hypaque [Pharmacia, Piscataway, N.J.] and 85% Hypaque) at 500 × *g* for 30 min at room temperature, as previously described (2). All subsequent procedures were carried out at 4°C. PMNs were collected and washed with Dulbecco's phosphate-buffered saline plus calcium (DPBS+) (Gibco, Grand Island, N.Y.), and any remaining erythrocytes were removed by hypotonic lysis. Cell suspensions were >95% PMNs by morphology after staining with Turks stain.

Lymphocyte isolation was performed by incubating the mononuclear band from the Ficoll-NIM gradient on an albumin-coated petri dish at 37°C for 2 h. The plates were rinsed with DBPS+, and the nonadherent leukocytes were discarded. The adherent lymphocytes were removed by gentle agitation with a Pasteur pipette.

PMNs were resuspended in RPMI 1640 culture medium (Gibco) containing 25 mM HEPES at pH 7.4. Incubations with inflammatory stimuli were performed

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with 10^7 PMNs in 3 ml of RPMI medium for 30 min at 37°C in an incubator with 5% CO_2. Cultures were prepared containing *Escherichia coli* 055:B5 LPS (Difco, Detroit, Mich.) and 1% normal pooled AB serum, fMLP (Calbiochem, La Jolla, Calif.), PMA, or A23187. At the end of incubation the supernate was aspirated and stored at −80°C and the cells were washed with DPBS+. PMN recovery was between 80 and 90%, and viability was >95%, as determined by trypan blue dye exclusion.

Receptor labeling. A total of 10^6 PMNs were incubated in DPBS+–0.1% gelatin–0.1% sodium azide (DPBS++) with an optimized quantity of phycoerythrin (PE)-conjugated anti-CD11b monoclonal antibody (MAb) (clone 2LPM19c; DAKO, Carpinteria, Calif.) or PE-conjugated anti-CD14 MAb (clone RM052; GenTrak, Plymouth Meeting, Pa.) for 30 min at 4°C in a final volume of 200 μl (28). The amount of antibody employed was determined by titration to provide maximal fluorescence of normal and stimulated PMNs and was approximately 1 μg/10^6 cells. Nonspecific antibody Fc binding was blocked with 10 μg of human gamma globulin. Isotypically matched PE-labeled monoclonal antibodies with no known specificity for human cells were used as negative controls. After incubation, cells were washed with DPBS++ and then pelleted by centrifugation at 500 × *g* for 5 min. The PMNs were fixed in 500 μl of a 1% paraformaldehyde solution and stored at 4°C.

Intracellular receptor staining. Fixation of PMNs and lymphocytes was accomplished by incubating the cells in 1 ml of a 1% paraformaldehyde solution at 4°C for 1 h. Plasma membranes then were made permeable by incubation at 4°C for 10 min in 2 ml of a 1% Triton X-100-based detergent. To remove the detergent, the cells were washed with 10 ml of DPBS and centrifuged at 500 × *g* for 10 min a total of three times. Then 10^6 PMNs were placed in DPBS++–1% gelatin–1% sodium azide buffer. Both human and mouse gamma globulin (100 μg each) and 20 μl of 0.2 M lysine solution were added to block nonspecific antibody binding. Fluorescein isothiocyanate-labeled anti-CD14 antibody (My4; Coulter, Hialeah, Fla., or Mo2; GenTrak) was then added, and the mixture was incubated at 4°C for 1 h in a final incubation volume of 200 μl. After incubation the cells were washed with DPBS++ and centrifuged. The PMNs were then resuspended in 3 ml of DPBS++ and again incubated at 4°C for 1 h to allow unbound anti-CD14 antibody to diffuse out of the cells. Cells were centrifuged and resuspended in 0.5 ml of 1% paraformaldehyde solution for storage until flow cytometry. Some PMNs that had undergone intracellular staining with a fluorescein isothiocyanate-labeled anti-CD14 MAb (as described above) were evaluated for the presence of intracellular fluorescence with an MRC 600 Nikon Diaphot confocal fluorescent microscope (Bio-Rad, Richmond, Calif.). Excitation occurred at 488 nm, and emission was measured at 520 nm.

Flow cytometry. Immunofluorescence studies were performed using an Epics 753 flow cytometer (Coulter) with the 488-nm line of an argon ion laser (3). Forward-angle light scattering and 90° light scattering as well as orange fluorescence were quantified. Electronic gates (bit maps) were set to include the granulocytes and exclude the mononuclear cells, platelets, debris, dead cells, and any remaining erythrocytes. At least 10,000 events were counted within the gated region. The orange fluorescence from PE was quantified after passing through a band pass filter (575 ± 15 nm) with linear amplification on a 256 channel scale. Microbead reference standards (Flow Cytometry Standards Corporation, Research Triangle Park, N.C.) were used to adjust photo multiplier tube voltages by using predetermined benchmark channels for the fluorochrome (38). Histograms were analyzed using Easy 2 software programs (Coulter). All flow cytometry results are presented as means ± standard errors of the mean (SEM).

PMN granule separation. PMNs were isolated from healthy human volunteers as previously described. PMN granules were released and separated by the method of Borregaard et al. (6). Briefly, isolated PMNs underwent nitrogen cavitation at 350 lb/in^2 for 30 min at 4°C. Whole PMNs and nuclei were removed by centrifugation of the cavitate at 500 × *g* for 10 min at 4°C. The cavitate was placed atop either a two-layer Percoll gradient for electron microscopy studies or a discontinuous sucrose gradient (20, 40, and a bumper of 60%) for all other studies. The densities of these sucrose solutions, except for the bumper, are approximately equal to the densities of Percoll utilized by Borregaard et al. (1.050, 1.120, and 1.122 g/ml). The samples were centrifuged at 6,000 rpm in a JS 13.1 rotor (Beckman Instruments, Fullerton, Calif.) for 2 h at 4°C for the sucrose gradient or as described by Borregaard et al. for the Percoll gradient. This method resulted in the standard three bands (membranes and secretory vesicles at the cavitate-20% interface, specific granules at the 20 to 40% interface, and azurophilic granules at the 40 to 60% interface).

CD14, β-glucuronidase, and B_{12}-binding protein assays. The concentration of CD14 was determined with a commercially available ELISA (Immuno Biological Laboratories, Hamburg, Germany) as previously described (17). The assay utilizes a horseradish peroxidase enzyme with an *o*-phenylenediamine substrate. Absorbance was read at 492 nm (microplate autoreader EL 309; Bio-Tek Instrument, Winooski, Vt.). The specificity of this assay was confirmed by using the kit's standard curve and a demonstration that the ELISA reaction could be blocked by the addition of excess LPS. The β-glucuronidase assay was performed with a commercially available kit (Sigma). This standard colorimetric assay was read on a DU-70 spectrophotometer (Beckman Instruments). B_{12}-binding protein assays were performed by the method described by Gallin et al. and Gottleib et al. (12, 14). The Co^{57} vitamin B_{12} was obtained from Diagnostics Products Corporation (Los Angeles, Calif.). Gamma radiation was quantified with a Micromedic Systems Automatic Gamma Counter 4/600 (Horsham, Pa.).

TABLE 1. CD14 and CD11b/CD18 expression on PMNs[a]

Exptl condition	Receptor expression on[b]:			
	Whole-blood PMNs		Isolated PMNs	
	CD14	CD11b/CD18	CD14	CD11b/CD18
Baseline	52 ± 8	150 ± 48	58 ± 3	242 ± 20
CA	65 ± 9	481 ± 102	74 ± 6	689 ± 25
LPS	93 ± 19*	2,295 ± 108*	112 ± 13*	1,908 ± 291*
fMLP	93 ± 3*	2,128 ± 76*	102 ± 15*	2,556 ± 141*

[a] Whole-blood samples or isolated PMNs were incubated in RPMI medium for 30 min at 37°C with no inflammatory agent (culture alone [CA]) or with LPS (10 μg/ml) or fMLP (10^{-5} M). Receptor expression, as MCF, was measured before (baseline) and after these incubations (data are means ± SEM).

[b] *, significant increase in receptor expression compared to baseline ($P < 0.05$ by RM-ANOVA [$n = 4$]). There were no significant differences between the values for whole-blood PMNs and isolated PMNs.

Electron microscopy. PMN granules were separated from PMNs as described above. The specific and azurophilic granule layers were aspirated and underwent high-speed centrifugation (13,000 × *g*) at 4°C for 5 min to pellet the granules. The granule pellets then were resuspended in 100 μl of 1% paraformaldehyde solution for 1 h. The CD14 staining of PMN granules was then performed in a manner similar to the PMN intracellular staining technique described above, with the exceptions that 200 μl of detergent was used to make the granules permeable, granules were pelleted by using an ultracentrifuge for 5 min at 4°C, and 100 μl of each anti-CD14 MAb (Coulter and GenTrak) was used during the antibody incubation. Gold labeling of the anti-CD14 MAb and electron microscopy were performed as described previously (32).

RESULTS

Surface CD14 receptor expression. As previously described, CD14 is present on the surface of most nonlymphoid leukocytes. Studies have shown that CD14 is present on the plasma membrane of approximately 20 to 40% of PMNs. In the present study, CD14 expression was evaluated for both whole-blood and isolated PMNs. CD14 was found to be constitutively expressed on 51% ± 4% (mean ± SEM, $n = 4$) of whole-blood PMNs. This percentage is similar to the 20 to 40% cited above. However, after PMN isolation the constitutive CD14 expression increased to 92% ± 2% of PMNs, suggesting the isolation procedure we employed induced an increase in CD14 expression. It is also possible that the isolation procedure resulted in the loss of some CD14 molecules from the cell surface or intracellular stores; however, this could not be quantified.

In addition to the constitutive expression of receptors the quantity of many plasma membrane proteins can be altered after exposure to immunostimulants. We investigated the response of whole-blood and isolated PMN CD14 expression to the immunostimulants LPS and fMLP. CD14 mean channel fluorescence (MCF), an indicator of receptor density, significantly increased after incubation of whole-blood and isolated PMNs with fMLP or LPS (Table 1). As noted above, the percentage of PMNs positive for CD14 was always greater than 90% for isolated PMNs. Despite this upregulation of CD14 by the isolation process, the ability of PMNs to increase CD14 MCF after stimulation with LPS or fMLP was not diminished. Because LPS and fMLP stimulation of PMNs results in the increased expression of CD11b/CD18 (30, 36, 43, 45) we employed CD11b/CD18 as a positive control for LPS and fMLP stimulation. In these studies both compounds, as expected, increased CD11b/CD18 MCF equally for whole-blood and isolated PMNs.

Intracellular CD14 staining. In an attempt to demonstrate the presence of intracellular CD14, PMNs were made permeable with Triton X-100-based detergent, incubated with a flu-

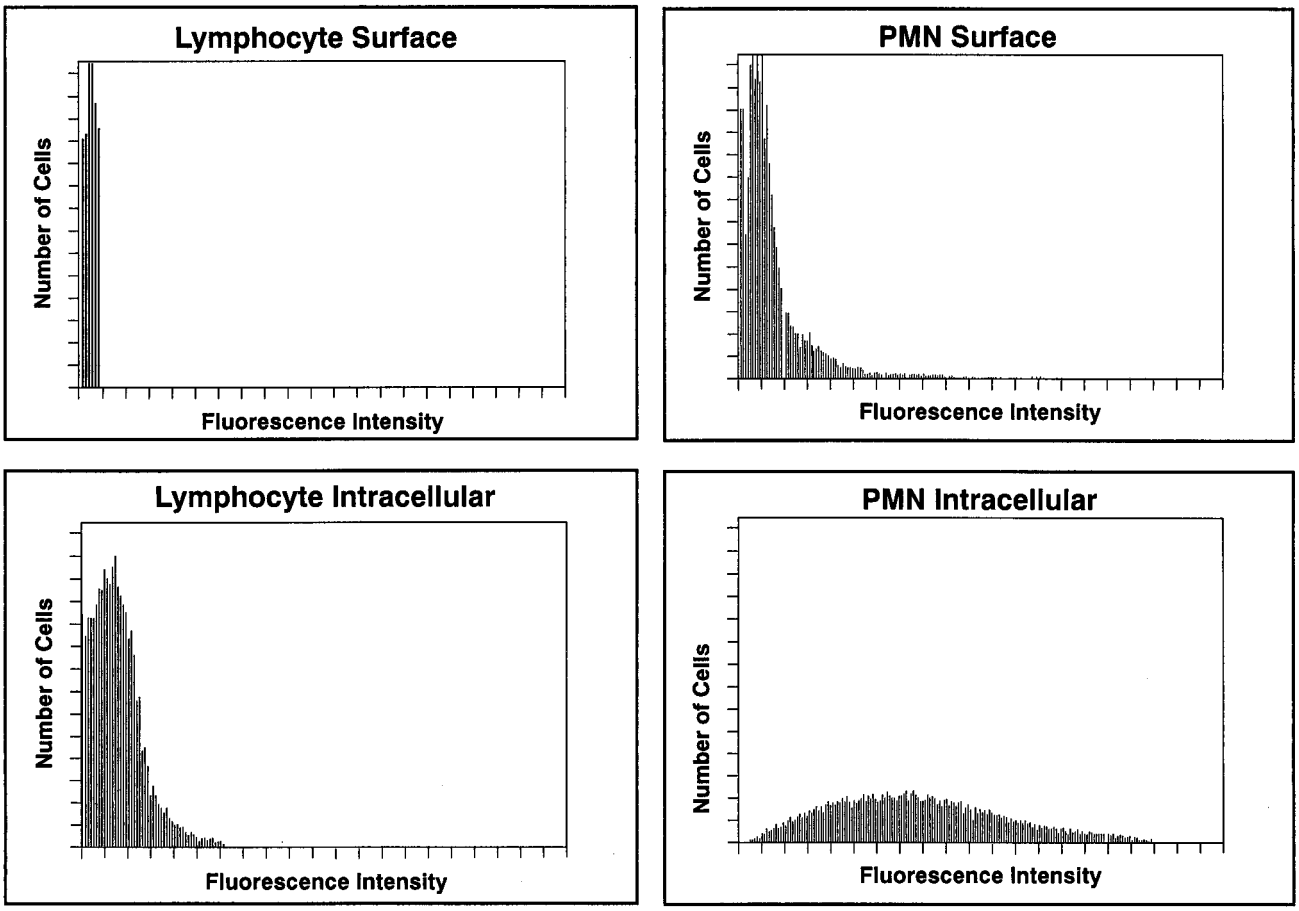

FIG. 1. CD14 expression in PMNs and lymphocytes. Flow cytometry was used to evaluate CD14 expression on the plasma membrane of lymphocytes and PMNs. After lymphocytes and PMNs were made permeable with a mild detergent, intracellular CD14 expression was also quantified by flow cytometry.

orescent-labeled anti-CD14 MAb (MY4 or Mo2), and observed by flow cytometry. These experiments demonstrated CD14 fluorescence in 93% ± 2% (n = 9) of permeable PMNs (Fig. 1). The specificity of antibody binding was investigated by three techniques. First, the fluorescence intensity distribution curves of these antibodies were easily distinguishable from those of isotypic controls, indicating that fluorescence was due to specific interaction within the PMN. Second, after isolation, both PMNs and lymphocytes were permeabilized and evaluated for the presence of intracellular CD14. T cells do not express CD14 and, therefore, provided a control for nonspecific binding. As predicted, the lymphocytes did not exhibit any noticeable fluorescence either on the plasma membrane of nonpermeabilized cells or within the cytoplasm of permeablized cells (Fig. 1). Finally, binding of the fluorescent-labeled antibody was blocked by preincubating the PMNs with a 100-fold excess of unlabeled antibody, resulting in a significant reduction of the CD14 MCF (Fig. 2). Binding was not completely blocked with the 100-fold excess of unlabeled antibody, indicating there was some nonspecific binding of the labeled antibody. The difference in CD14 fluorescence intensities between the two antibodies, MY4 and Mo2, was most likely related to Mo2 being an immunoglobulin M antibody with more fluorescent molecules attached.

To ensure that the fluorescence being measured was originating from the cytoplasm, a fluorescent confocal microscope was used to optically section the PMN cytoplasm at multiple levels (Fig. 3). The fluorescence displayed a granular cytoplasmic distribution, was not present within the nucleus, and was present in all PMNs observed.

PMN CD14 expression and release of sCD14. To localize the intracellular pool of CD14 we initially looked at the immunostimulants PMA and A23187, which cause the mobilization of specific but not azurophilic granules (44). Isolated PMNs were

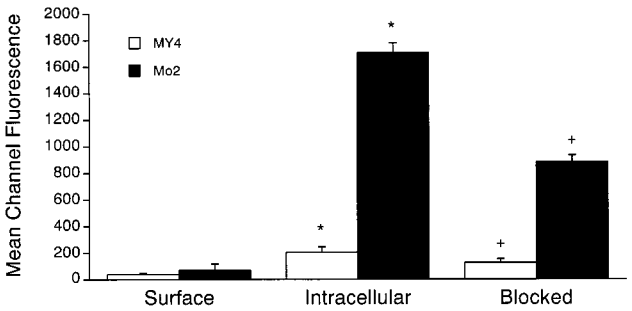

FIG. 2. Intracellular CD14 expression in PMNs. Whole and permeable cells were incubated with two anti-CD14 antibodies (MY4 and Mo2). Labeled antibody binding was blocked with a 100-fold excess of unlabeled antibody. CD14 receptor presence on the cell surface and intracellularly was evaluated by flow cytometry (data are means ± SEM). *, significant difference from surface CD14 MCF; +, significant difference from intracellular CD14 MCF. ($P < 0.05$ by Student's t test [n = 9]).

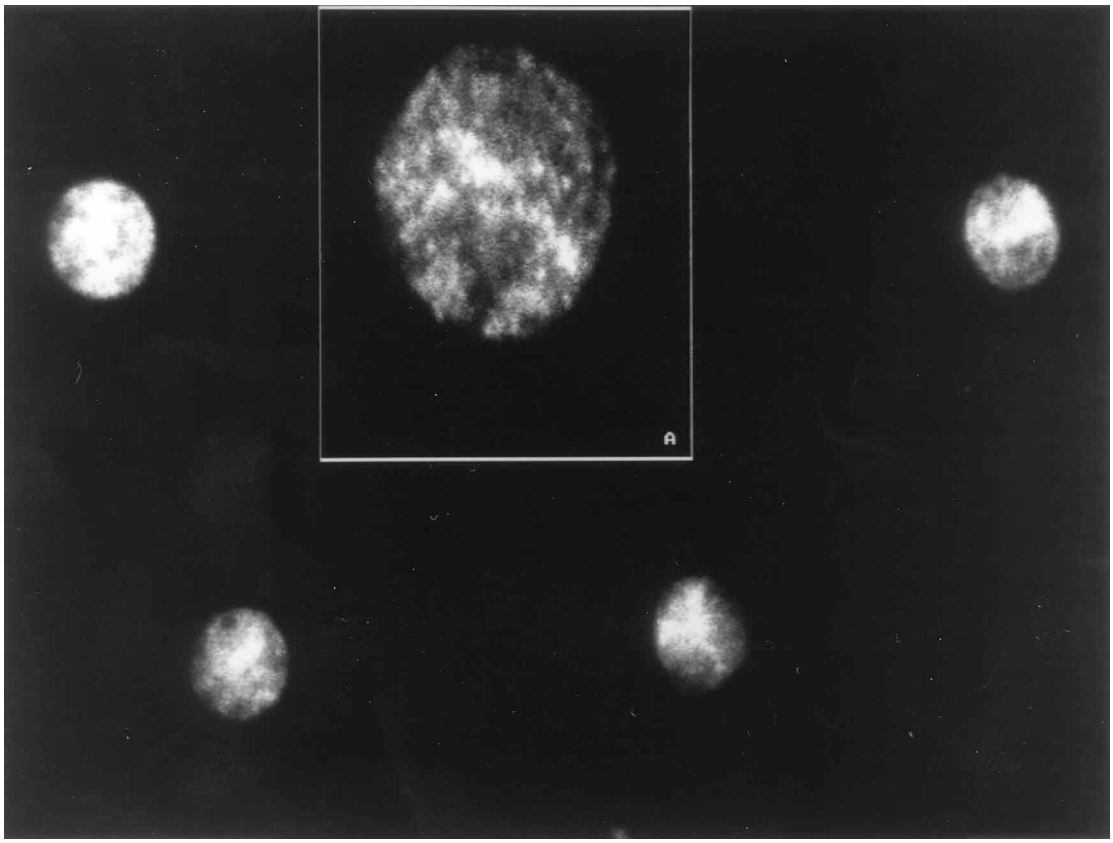

FIG. 3. Intracellular CD14 evaluation by confocal microscopy. Permeabilized PMNs, incubated with anti-CD14 antibody (My4), were evaluated for the presence of intracellular CD14 by confocal microscopy. The micrograph shows four PMNs with intracellular fluorescence. The inset is a magnified view of a single PMN.

stimulated with PMA or A23187, and the expression of CD14 was quantified. Neither PMA or A23187 increased the CD14 MCF (Table 2). Similar to LPS and fMLP, both PMA and A23187 increase the expression of CD11b-CD18 on the surface of normal human PMNs (10). Again, in these experiments, measurement of CD11b-CD18 MCF was used as a positive control. Both PMA and A23187 stimulated PMN CD11b-CD18 upregulation appropriately (Table 2).

A parallel ELISA quantification of sCD14 present in the culture medium from these samples also was performed. Neither PMA or A23187 caused a release of sCD14 (Table 2). Phospholipase C treatment of PMNs leads to the release of sCD14 (21), and when used in our culture conditions, such treatment demonstrated that if CD14 had been released from the plasma membrane, or from intracellular pools by exocytosis, it would have been detected by the sCD14 ELISA. Exposure of the PMNs to phospholipase C (0.2 U/ml) for 30 min at 37°C in RPMI culture medium resulted in a significant decrease in the percentage of CD14-positive PMNs from 90.1% ± 2% to 26% ± 10% and a significant increase in sCD14 from 14.9 ± 0.9 to 48.5 ± 2.1 ng/ml ($P < 0.05$ by repeated-measurements analysis of variance [RM-ANOVA]; $n = 3$).

To further evaluate the response of CD14 to LPS and fMLP we measured PMN CD14 MCF and the quantity of sCD14 after incubation with these immunostimulants in conditions similar to the experiments above. As previously demonstrated fMLP was able to increase the CD14 MCF from 178 ± 7 to 231 ± 8 ($P < 0.05$ by RM-ANOVA; $n = 4$); however, the sCD14 ELISA absorbance did not significantly change (293 ± 3 versus 306 ± 17). The same experiment was attempted with LPS; however, the LPS in the culture medium interfered with the sCD14 ELISA (data not shown).

Subcellular CD14 localization. Both PMA and A23187 induce the exocytosis of specific granules, and as presented in the previous section, neither altered observed CD14 expression. To determine if azurophilic granules contained CD14, PMN granules were released by cavitation and separated by centrifugation on a discontinuous sucrose gradient. This method of isolation was effective in separating the different types of granules, as evidenced by assays measuring the amount of B_{12}-binding protein (specific granules) and β-glucuronidase (azurophilic granules). The B_{12}-binding protein content of the

TABLE 2. Measurement of CD14 MCF, sCD14, and CD11b/CD18 MCF after incubation with PMA or A23187[a]

Exptl condition	Receptor expression on PMNs[b]		
	CD14 MCF	sCD14 (ng/ml)	CD11b/CD18 MCF
CA	137 ± 5	14.9 ± 0.9	984 ± 262
PMA	148 ± 8	22.9 ± 2.9	2,066 ± 236*
A23187	133 ± 17	24.0 ± 3.3	6,637 ± 152*

[a] PMNs were incubated in RPMI culture medium alone (CA) or with PMA (40 nM) or A23187 (10^{-6} M) for 30 min at 37°C. Receptor expression on PMNs and quantification of sCD14 in the medium was determined by flow cytometry and ELISA, respectively (data are means ± SEM). No significant differences in CD14 expression or sCD14 quantification were noted among PMNs incubated with CA, PMA, or A23187.

[b] *, $P < 0.05$ by RM-ANOVA ($n = 4$).

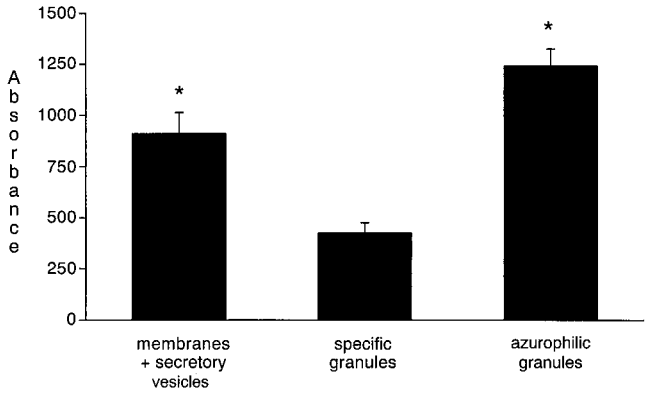

FIG. 4. Presence of CD14 in PMN granules. PMNs underwent cavitation to release granules that were then separated by a discontinuous sucrose gradient. CD14 content in each of the different granules was evaluated by ELISA absorbance (data are means ± SEM). *, significant difference from specific granules ($P < 0.05$ by RM-ANOVA [$n = 5$]).

specific granule layer was 80% of the total, and the β-glucuronidase content of the azurophilic granule layer was 76% of the total ($n = 3$). The amount of CD14 in each of the granular layers was quantified utilizing an ELISA. By this method, the CD14 content was slightly greater in the azurophilic granule layer than the plasma membrane-secretory vesicle layer. However, both of these layers contained significantly more CD14 than the specific granule layer (Fig. 4).

To further confirm these results electron microscopy was used to evaluate the presence of CD14 within the different types of PMN granules. Utilizing a discontinuous Percoll gradient the specific and azurophilic granules were isolated from cavitated PMNs. The granules then were made permeable by a method similar to that employed for the whole-blood PMN studies. These granules then were incubated with gold-conjugated anti-CD14 antibody (My4) and evaluated utilizing electron microscopy. Electron photomicrographs demonstrated the presence of gold particles within the azurophilic but not the specific granules, thereby confirming the ELISA results (Fig. 5).

DISCUSSION

The interaction between LPS, in conjunction with LBP (40), and CD14 is the first step in leukocyte activation (34, 46). To review, the results of this study demonstrated that CD14 is present in the cytoplasm of most PMNs and that CD14 can be rapidly mobilized to the cell surface following immunostimulation by fMLP and LPS but not by PMA or A23187. None of these proinflammatory agents induced the release of sCD14. Cytoplasmic CD14 appears to reside within PMN azurophilic granules, in addition to being within the previously described secretory vesicles (7).

In addition to its effect on CD14 expression, isolation leads to upregulation of other cell surface proteins (42). Of interest is the observation that the isolation process increases the expression of CD16 (42). If, as proposed by Detmers et al. (7), CD14 is stored within the same secretory vesicles as CD16 then it follows that if the isolation procedure increases the cell surface expression of CD16 it also should increase that of CD14. Even though CD14 was detected on a larger percentage of PMNs after isolation, the isolation process itself did not appear to alter the ability of PMNs to increase the density of CD14 cell surface expression, as determined by CD14 MCF, after exposure to LPS and fMLP.

The results of CD14 expression on PMNs and upregulation with LPS or fMLP are consistent with most previously published reports (1, 43, 45); however, not all studies have shown that LPS increases CD14 expression (45). Such discrepancies may be attributable to differences in incubation conditions, antibody selection, and flow cytometry methodologies employed by each group. Weaker immunostimulants, such as LPS and fMLP, preferentially cause the mobilization of receptors from secretory vesicles to the plasma membrane (36). The observation that LPS and fMLP increase CD14 cell surface expression on PMNs supports the observations of Detmers et al. (7) that preformed CD14 is located within secretory vesicles. Our results that fMLP does increase CD14 expression but does not induce the release of sCD14 also support the idea that this intracellular pool is not released by exocytosis to increase levels of sCD14 but instead increases the plasma membrane CD14 expression. The increase in CD14, after stimulation with LPS or fMLP, was smaller than the increase in CD11b-CD18. The exact physiologic significance of these changes is uncertain.

PMA and A23187 preferentially mobilize specific, but not azurophilic, granules resulting in the exocytosis of enzymes and upregulation of plasma membrane proteins stored within specific granules (36, 44). The observation that neither PMA or A23187 increases CD14 cell surface expression, nor causes the release of sCD14, suggests that CD14 is not present within specific granules. The amount of CD14 present in the specific granules, detected by the ELISA, is consistent with the 20 to 25% granule impurity after separation determined by the B_{12}-binding protein and β-glucuronidase assays.

The observation that nearly all PMNs contain intracellular pools of CD14 is not surprising. All PMNs need to be able to respond to the LPS molecules associated with gram-negative infections, a response that is dependent upon CD14 (43, 45). If the CD14 stored within secretory vesicles is involved in receptor upregulation what is the role of the CD14 present within the azurophilic granules? Azurophilic granules contain proteases and bactericidal proteins, do not readily undergo exocytosis, and are involved with phagocytosis by acting as a lysozome. It has been proposed that CD14 not only acts in LPS signal transduction but also is involved in the rapid uptake of LPS into leukocytes (29, 41). After LPS binds to CD14 the complex can be internalized (11, 16, 25, 26, 29) and then the internalized LPS undergoes deacylation (29). This process also extends to intact bacteria (23, 45, 47). The mechanism for CD14-LPS internalization is unknown but may involve potocytosis (29). Potocytosis is the process by which glycosylphosphotidylinositol-anchored receptors are internalized as caveolae. We speculate that the CD14 present in azurophilic granules may originate from the internalized CD14-LPS complex.

Another possibility is that azurophilic-stored CD14 may be released extracellularly during PMN destruction and be used to modify the inflammatory response as sCD14. When PMNs are in an area of inflammation-infection they frequently lyse, resulting in the release of enzymes and proteins (22), likely including CD14. Studies have demonstrated that sCD14 can insert into the plasma membrane of cells, making these cells more sensitive to LPS stimulation. For example, when endothelial cells, which do not express CD14, are exposed to LPS in the presence of sCD14, they synthesize and release proinflammatory cytokines and express adhesion receptors (9, 13, 33). Also, PMNs can undergo activation when they are incubated with the LPS-sCD14 complex (18). These interactions of sCD14 with endothelial cells and PMNs result in potentiation of the inflammatory response. Most studies, however, have demonstrated that for cells normally expressing CD14 the

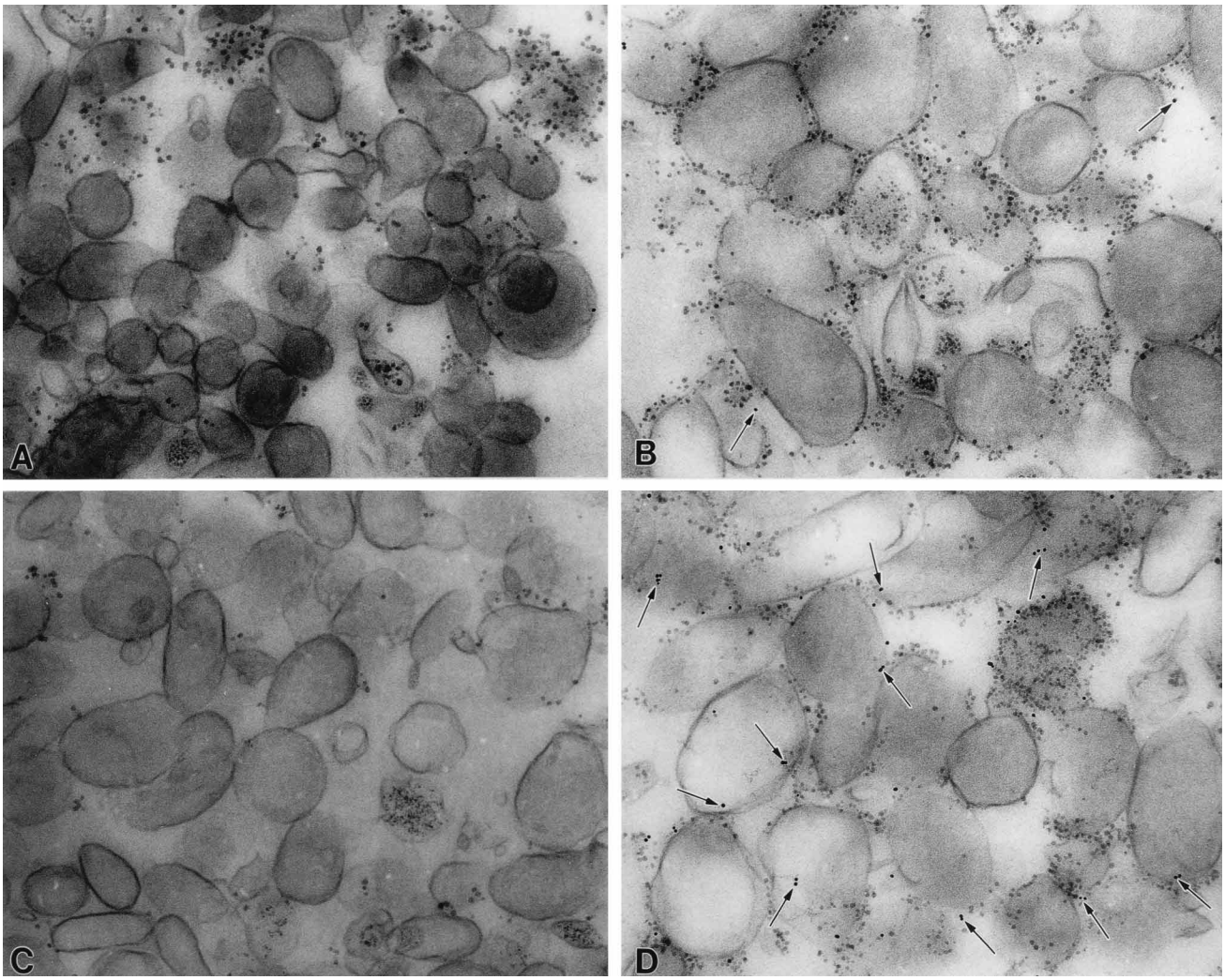

FIG. 5. Electron microscopic evaluation of CD14 in granules. PMNs underwent cavitation to release granules that were then separated by a discontinuous Percoll gradient. CD14 content for the different granules was evaluated by electron microscopy. (A) Specific granules and no anti-CD14 antibody; (B) specific granules with anti-CD14 antibody; (C) azurophilic granules and no anti-CD14 antibody; (D) azurophilic granules with anti-CD14 antibody. Arrows indicate examples of specific CD14 staining.

presence of sCD14 binds free LPS, thereby acting as a down-regulatory mechanism (19). Support for this hypothesis is found in other studies that suggest that LPS-sCD14 complexes can be inert and may not lead to leukocyte activation (35). Also, sCD14 binds to intact gram-negative bacteria, thereby aiding in the elimination of these organisms (23). Although the final effect of these sCD14 interactions are unclear, infusion of sCD14 prevents LPS-induced mortality in mice, implying a beneficial effect to the host from the release of sCD14 (20).

As noted above some of our results support the results of the study by Detmers et al. (7), specifically those suggesting that CD14 is located within secretory vesicles and that this pool does not undergo exocytosis to provide sCD14. In addition, our results show that CD14 is probably not present to any significant degree within specific granules. However, our results demonstrate that in addition to secretory vesicles a significant portion of the PMN intracellular CD14 is present within azurophilic granules. The most likely explanation for this difference between the studies concern the methods used for granule separation. We utilized a discontinuous sucrose gradient as opposed to the discontinuous Percoll gradient used by Detmers et al. We found that the high concentration of Percoll associated with the azurophilic granules frequently interfered with many of our assays and this problem was alleviated with the use of a sucrose gradient.

In conclusion, the results of this study demonstrate that CD14 is present on the surface of PMNs and can be rapidly increased following immunostimulation by fMLP and LPS but not by PMA or A23187. None of these immunostimulants induced the release of sCD14. We showed that CD14 is present within the cytoplasm of PMNs and appears to reside within the azurophilic granules and secretory vesicles.

ACKNOWLEDGMENTS

We thank Susan Dawes for her help with flow cytometry.

This work was supported, in part, with grants from the Shriners of North America and the Surgical Infection Society.

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Editor: R. E. McCallum