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Heterogeneity in Lewis-X and Sialyl-Lewis-X Antigen Expression on Monocytes in Whole Blood

Relation to Stimulus-Induced Oxidative Burst

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By using flow cytometric analysis of cells in whole blood expressing high levels of CD14, we found a subpopulation of monocytes (8% of total) with higher scatter parameters, high capacity to produce reactive oxygen species (ROS), stronger expression of Lewis-X (CD15), sialyl-Lewis-X, CD11b and CD18 antigens, as well as an increased polymerized actin content. The size of this subpopulation increased after stimulation with lipopolysaccharide at the expense of the remaining monocytes, suggesting that its features were inducible. The membrane increase in Lewis-X and sialyl-Lewis-X expression observed during this conversion was largely due to the translocation of these carbohydrate structures from intracellular pools. Moreover, this subpopulation behaved as a primed monocyte subpopulation producing large amounts of H_2O_2 in response to *N*-formyl-methionyl-leucyl-phenylalanine. Increased H_2O_2 production was inhibited not only by anti-CD14 but also by anti-CD15 and anti-sialyl-Lewis-X monoclonal antibodies when added before lipopolysaccharide. These results show that lipopolysaccharide priming is regulated, at least in part, by Lewis-X and sialyl-Lewis-X structures expressed on the monocyte membrane. All together, this highly reactive and inducible subpopulation of monocytes, which share phenotypic and functional characteristics with neutrophils, might play an important role in host defenses and inflammatory responses. *(Am J Pathol 1998, 152:1081–1090)*

Human monocytes play a critical role in host defenses against invading microorganisms.[1,2] In response to pathogens, monocytes move from the circulating blood to the infected tissues where their activation leads to the production of reactive oxygen species (ROS) in the so-called oxidative burst. It is generally considered that monocytes produce less ROS than polymorphonuclear neutrophils (PMN).[3] Human blood monocytes, however, are heterogeneous in terms of cell volume,[4] phagocytosis,[4,5] CD14 and CD16 expression, and cytokine production.[6,7] In addition, a discrete subpopulation of human monocytes shows a strong capacity to produce ROS and strong expression of β2 integrins,[8,9] which are involved in adherence to endothelial cells and in transendothelial migration.[10,11] The Lewis-X (3-α-fucosyl-*N*-acetyl-lactosamine, Le^X) and its sialylated derivative, sialyl-Lewis-X (sLe^X), also participate in the complex process of adhesion between leukocytes and endothelial cells, in particular involving the selectin adhesion molecules.[12–14] These carbohydrate structures are expressed on several glycoproteins and glycolipids of the leukocyte membrane.[15–17] Lewis-X antigen, which is recognized by CD15 monoclonal antibodies, is considered to be present at a low level on monocytes,[18] whereas it is a characteristic phenotypic marker of PMN.[19,20] Sialyl-Lewis-X structures are present on both monocytes and neutrophils. Possible monocyte heterogeneity in their expression of Lewis-X and sialyl-Lewis-X has never been investigated.

It is not clear whether or not the monocyte population with high ROS production[8,9] represents an inducible subpopulation. It has repeatedly been shown that PMN can be primed for their oxidative burst;[21] this priming process is a mechanism whereby resting PMN acquire a state of preactivation that leads to a more powerful bactericidal response at the inflammatory site. This priming process has been demonstrated *in vitro* by pretreatment of PMN with a number of cytokines, endotoxin,[22–24] and adhesion molecules such as β2 integrins.[25,26] This phenomenon has also been reported for monocytes[27,28] but has not been analyzed in terms of membrane expression of adhesion molecules and their functional involvement in ROS production.

Previous studies of monocyte heterogeneity were done on monocytes isolated from other blood cells by various procedures that may differently modulate surface receptor expression and alter cell responses.[22,29] The aim of this study was to analyze the phenotypic and functional heterogeneity of monocytes by using whole blood to

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minimize procedure-related changes in surface receptor expression, an approach we have previously used to study PMN.[22,30] Flow cytometry, which can be used to study events at the single-cell level, was chosen to identify monocytes in terms of high CD14 expression ($CD14^{high}$ cells) and to measure some key factors in monocyte bactericidal activity, ie, 1) ROS production focusing on H_2O_2 production in response to bacterial *N*-formyl-peptides (*N*-formyl-methionyl-leucyl-phenylalanine) after lipopolysaccharide (LPS) priming, 2) expression of the β2 integrin CD11b/CD18 and the carbohydrate structures Lewis-X and sialyl-Lewis-X, and 3) actin polymerization that appears to be involved in both receptor mobilization and the oxidative burst. In addition, as Lewis-X (CD15) and sLe^X carbohydrate structures have been implicated in the neutrophil oxidative burst,[31,32] we analyzed the involvement of Le^X and sLe^X antigen expression in monocyte ROS production in response to bacterial stimuli.

Materials and Methods

Reagents

The reagents and sources were as follows: 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) (Eastman Kodak, Rochester, NY); endotoxin (LPS) from *Escherichia coli* (055 = B5), fluorescein-LPS from *E. coli* (055 = B5), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), unlabeled phalloidin, fluorescein isothiocyanate (FITC)-phalloidin, L-α-lysophosphatidylcholine, cycloheximide, and cytochalasin B (Sigma, St. Louis, MO); phycoerythrin (PE)-conjugated mouse monoclonal IgG2b anti-human CD14 antibody (PE-anti-CD14), fluorescein-conjugated mouse monoclonal IgG2a anti-human HLA-DR (FITC-HLA-DR) and IgG1 anti-human CD45 (FITC-CD45) antibodies, mouse monoclonal IgM antibodies to human Lewis-X (anti-CD15) and sialyl-Lewis-X (anti-sLe^X), mouse monoclonal IgG1 antibodies to CD18 (anti-CD18) and platelet-endothelial cell adhesion molecule (PECAM) (anti-PECAM), mouse monoclonal IgG2b antibody to CD14 (anti-CD14), and labeled and unlabeled mouse isotype controls for IgG1, IgG2a, and IgG2b antibodies (Becton Dickinson Immunocytometry Systems, San Jose, CA); fluorescein-conjugated mouse monoclonal IgM anti-human CD15 antibody (FITC-anti-CD15) and labeled and unlabeled mouse isotype controls for IgM antibodies (Dakopatts, Glostrup, Denmark); fluorescein-conjugated mouse monoclonal IgG1 anti-human CD11b antibody (FITC-anti-CD11b), anti-human CD18 (FITC-anti-CD18), and mouse isotype control for IgG1 antibodies (Immunotech, Marseille, France); mouse monoclonal IgG1 antibody to human L-selectin (anti-LAM) (Coultronics, Hialeah, FL); FITC-goat anti-mouse immunoglobulins (FITC-goat anti-mouse immunoglobulins) (TEBU, Santa Cruz, CA); FITC-goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL); fetal calf serum (Life Technologies, Inc., Grand Island, NY); and liquemine (Panpharma, Fougeres, France).

Stock solutions of DCFH-DA (50 mmol/L) and fMLP (10^{-2} mol/L) were prepared in dimethylsulfoxide and stored at −20°C. The solutions were diluted in phosphate-buffered saline (PBS; Pharmacia, Uppsala, Sweden) immediately before use.

Incubation of Whole Blood with LPS and Formyl Peptides

Fresh blood (1 ml) from healthy donors collected onto preservative-free liquemine (10 U/ml blood) was incubated with LPS (0.01 to 25 μg/ml) diluted in PBS or with PBS alone for various times (15 to 120 minutes) in a 37°C water bath with gentle horizontal shaking. fMLP diluted in PBS (10^{-6} mol/L) or a similar dilution of dimethylsulfoxide was then added for 1 to 5 minutes at 37°C. Standard priming conditions were selected in preliminary studies and consisted of 30 minutes of preincubation with 5 μg/ml LPS followed by incubation with fMLP (10^{-6} mol/L) for 5 minutes. After incubation with LPS, fMLP, or control solutions, the reaction was stopped on ice and samples were incubated with PE-anti-CD14 antibody for 30 minutes at 4°C. Red cells were lysed using fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson Immunocytometry Systems), and white cells were suspended in 1% paraformaldehyde. FACS lysing solution neither modified the amount of dichlorofluorescein (DCF) generated nor increased the expression of activation markers such as CR3 as measured by flow cytometry (data not shown). Moreover, monocyte viability was not altered in our priming conditions as assessed in terms of propidium iodide exclusion by means of flow cytometry.

H_2O_2 Production

H_2O_2 production was measured by using a flow cytometric assay derived from the technique described by Bass et al.[33] Before treatment with LPS and/or fMLP, whole blood was incubated for 15 minutes with 2′,7′-DCFH-DA (100 μmol/L) in a 37°C water bath with gentle horizontal shaking. DCFH-DA diffuses into cells and is hydrolyzed into 2′,7′-dichlorofluorescin (DCFH); during the monocyte oxidative burst, nonfluorescent intracellular DCFH is oxidized into highly fluorescent DCF by H_2O_2.[33] In experiments in which the effects of specific monoclonal antibodies on the monocyte oxidative burst were examined, these reagents were added 5 minutes before incubation with LPS or, in other experiments, before fMLP. After incubation with anti-CD14 antibody as described above, red cells were lysed. The fixed samples were kept on ice until flow cytometric analysis on the same day.

F-Actin Content of Monocytes

Whole blood was either kept on ice or incubated at 37°C with LPS or PBS for 30 minutes as described above. After incubation with anti-CD14 antibody, white cells (obtained after red cell lysis) were fixed with 1% paraformaldehyde-PBS, and F-actin content was measured in a flow cyto-

metric assay.[34] The cell suspension (100 μl) was incubated for 30 minutes at 0°C in 100 μl of 8% paraformaldehyde and 200 μl/ml of L-α-lysophosphatidylcholine in PBS with or without 1 mmol/L unlabeled-phalloidin to measure nonspecific binding of FITC-phalloidin. FITC-phalloidin (20 μmol/L) was then added to the suspension, and incubation was continued for 30 minutes at 0°C. After one wash in PBS, the cells were resuspended in 1% paraformaldehyde-PBS.

Determination of Adhesion Molecule Expression at the Monocyte Surface

Whole blood was either kept on ice or incubated at 37°C with LPS (labeled LPS was used in certain experiments) or control solutions as described above. In some experiments, cycloheximide (20 μg/ml), cytochalasin B (5 μg/ml), or pentoxifylline (10 μmol/L) were added 5 minutes before treatment with LPS. To study Le^X antigen, CD11b and CD18 molecule expression on CD14^{high} cells, samples (100 μl) were incubated at 4°C for 45 minutes with the following monoclonal reagent combinations: FITC-anti-CD15/PE-anti-CD14, FITC-anti-CD11b/PE-anti-CD14, and FITC-anti-CD18/PE-anti-CD14. To study sLe^X antigen and L-selectin expression on CD14^{high} cells, samples were incubated with nonconjugated anti-sLe^X or anti-LAM (L-selectin) antibodies for 45 minutes at 4°C, washed with ice-cold PBS, and then incubated at 4°C for 45 minutes with FITC-GAM or FITC-goat-anti-mouse IgM; after one wash in ice-cold PBS, samples were incubated with PE-anti-CD14 at 4°C for 30 minutes. Red cells were lysed with FACS lysing solution, and white cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry. Nonspecific antibody binding was determined on cells incubated with the same concentration of an irrelevant antibody of the same isotype.

Determination of Intracellular Expression of Le^X and sLe^X Antigens

Whole blood (100 μl) was incubated with PE-anti-CD14 for 30 minutes at 4°C. Red cells were lysed with FACS lysing solution. White cells were washed twice with PBS containing 2% fetal calf serum. Paraformaldehyde (0.25%) was then added while vortexing, and samples were incubated in the dark for 15 minutes at room temperature. After one wash with PBS, cells were incubated with ice-cold PBS containing 70% methanol in the dark for 60 minutes at 4°C to permeabilize the cell membranes. After one wash in PBS, samples were incubated with FITC anti-CD15 and anti-sLe^X antibodies for 30 minutes at 4°C. To study sLe^X antigen expression, samples were then incubated with FITC-goat-anti-mouse IgM. After one wash with ice-cold PBS-fetal calf serum, cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometric analysis.

Flow Cytometric Analysis

We used a Becton Dickinson FACScan (Immunocytometry Systems, San Jose, CA) with a 15-mW, 488-nm argon laser. Anti-CD14 antibody was used to identify the monocyte population, which appeared on the PE-CD14 antibody fluorescence histogram as highly fluorescent CD14$^+$ cells (CD14^{high} cells). Ten thousand CD14^{high} cells were counted per sample, and fluorescence pulses were amplified by 4-decade logarithmic amplifiers. The green fluorescence of DCF, FITC-phalloidin, FITC-anti-CD15, FITC-anti-CD11b, FITC-anti-CD18, FITC-LPS, FITC-goat-anti-mouse IgM, and FITC-GAM was recorded from 515 to 545 nm, and the orange fluorescence of PE-anti-CD14 was recorded from 563 to 607 nm. In all cases, unstained cells were ran, and photomultiplier settings were adjusted so that the unstained cell population appeared in the lower left corner of the fluorescence display. Single-cell controls were used to optimize signal compensation. All of the results were obtained with a constant photomultiplier gain. The data were analyzed using LYSIS II software (Becton Dickinson Immunocytometry Systems), and the mean fluorescence intensity (MFI) was used to quantify the responses. The MFI of a matched isotype control was recorded and subtracted from the MFI obtained with the tested antibody. The MFI of nonspecific binding of FITC-phalloidin was subtracted from the value obtained with FITC-phalloidin. The effect of agonists on H_2O_2 production was calculated using a stimulation index (ratio of mean fluorescence intensity of stimulated cells to that of unstimulated cells). In some experiments, CD14^{high}/CD15$^+$ cells were sorted using a FACSsort (Becton Dickinson Immunocytometry Systems) and were collected for cytospin preparation and microscopic examination.

Statistical Analysis

Data are means ± SEM (SEM). Means were compared using Student's *t*-test, and *P* values of 0.05 or less were considered significant.

Results

Flow Cytometric Identification of Two Monocyte Subpopulations in Whole Blood and Their Interconversion after LPS Stimulation

Whole blood was incubated at 4°C with PE-anti-CD14 antibody for 30 minutes. Flow cytometric analysis of CD14^{high} cells distinguished, on the bivariate light scatter dot plot, two subpopulations that differed in forward and right-angle scatter. The first, large subpopulation (92% of CD14^{high} cells) grouped at lower scatter parameters than the second subpopulation (8% of CD14^{high} cells) (Figure 1, A and B). Dual-color immunofluorescence with anti-CD14 antibody, and either anti-CD11b or anti-CD18 antibody showed that the latter molecules were expressed at substantially higher levels in the second subpopulation

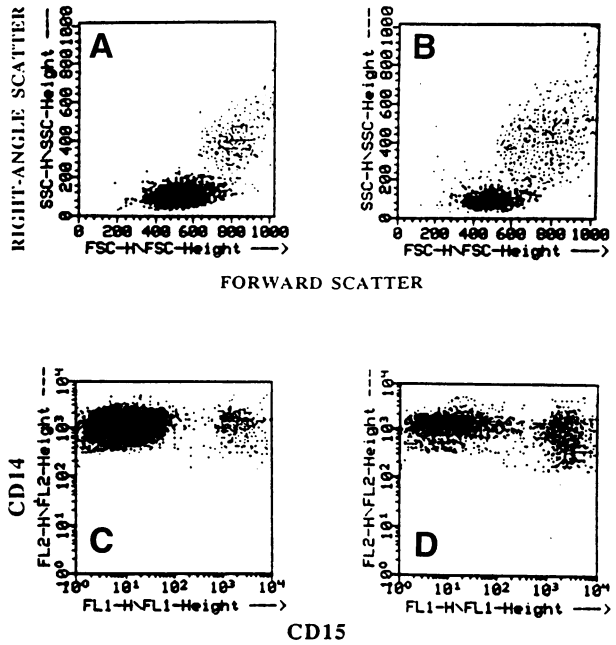

Figure 1. Flow cytometric identification of two monocyte subpopulations in whole blood. Whole blood was maintained at 4°C (**A** and **C**) or incubated with LPS (**B** and **D**). Flow cytometric analysis allowed us to distinguish two subpopulations that differed in forward and right-angle scatter on the bivariate light scatter dot plot (**A** and **B**). Lewis-X antigen (CD15) expression differed at the cell surface of these two subpopulations (**C** and **D**).

than in the first, as defined by scatter parameters. MFI on unstimulated cells maintained at 4°C was two-fold higher for the binding of anti-CD11b and anti-CD18 antibody on the second subpopulation than on the first (Table 1).

To gain additional information on the two CD14^{high}/ populations of monocytes, we studied the expression of other adhesion molecules at the cell surface. Le^X and sLe^X carbohydrate structures also showed differences between the two subpopulations (Table 1). Le^X antigen

Table 1. Identification of Two Subpopulations of Monocytes in Whole Blood†

Features of monocytes	Mean fluorescence intensity‡ First subpopulation (9 ± 2%)	Second subpopulation (8 ± 2%)
Anti-CD11b mAb	38 ± 2	58 ± 3*
Anti-CD18 mAb	31 ± 8	58 ± 12*
Anti-Le^x mAb	9 ± 2	736 ± 47*
Anti-sLe^x mAb	234 ± 42	1009 ± 75*
H_2O_2 production	23 ± 3	74 ± 12*
Polymerized actin	38 ± 8	153 ± 7*

†Whole blood was incubated at 4°C with PE-anti-CD14 antibody for 30 minutes. Flow cytometric analysis of CD14$^+$ cells distinguished two subpopulations whose percentages are given in brackets and which differed, on the bivariate light scatter dot plot, in forward and right-angle scatter. The subpopulation that exhibited an increase in cell size and granularity is referred to as the second subpopulation. Expression of CD11b, CD18, Lewis X (Le^x), sialyl-Lewis X (sLe^x), dichlorofluorescin diacetate oxidation into dichlorofluorescein, and actin polymerization were analyzed as described in Materials and Methods.

‡The mean fluorescence intensity (MFI) was recorded as described in Materials and Methods. The MFI of the isotypic controls was less than 5 and was subtracted.

*Significantly different from the first subpopulation ($P < 0.05$). Values are means ± SEM (n=10).

(CD15) membrane expression was barely detectable on the first subpopulation: the MFI with anti-CD15 antibody did not differ significantly from the isotypic control value. In contrast, Le^X was highly expressed (MFI = 736 ± 47) on the second subpopulation. Similarly, the MFI of sLe^X was fourfold higher in the second than the first subpopulation (Table 1). Unstimulated cells of the first subpopulation expressed low background fluorescence after DCFH-DA loading for 15 minutes at 37°C. In contrast, DCF fluorescence was significantly higher with unstimulated cells from the second subpopulation (Table 1). As also shown in Table 1, the MFI of FITC-phalloidin binding, a marker of polymerized actin, was significantly higher in the second subpopulation than in the first.

Flow cytometry thus clearly distinguished two monocyte subpopulations in unstimulated whole blood. To determine whether the two subpopulations were interconvertible states, whole blood was stimulated with LPS (5 μg/ml for 30 minutes) before analysis by flow cytometry. The second subpopulation of CD14^{high} cells expanded from 11% (PBS alone) to 46% (Table 2). This probably resulted from changes in the first subpopulation as the same total number of monocytes was analyzed and as CD14 expression was not altered after stimulation. The percentage of CD14^{high} cells among total leukocytes, measured by dual immunofluorescence staining with PE-CD14 and FITC-CD45 antibodies, was not modified after LPS stimulation as compared with control samples incubated with PBS alone. CD15 expression by cells remaining in the first subpopulation was barely modified after stimulation with LPS. In contrast, it was markedly increased after stimulation in the second subpopulation (Table 2; Figure 1, C and D). sLe^X antigen expression (MFI per cell) fell in the cells remaining in the first subpopulation and in cells belonging to the second subpopulation (Table 2). The latter subpopulation, however, was much larger than before stimulation. The MFI and percentage of members in each subpopulation can be combined in an index (MFI × %) representing all cell-surface expression of the molecule concerned. This index, calculated for Le^X and sLe^X, clearly increased in the second subpopulation of monocytes (Table 2, results in brackets). LPS added to whole blood induced an increase in CD11b and CD18, marked actin polymerization, and significant but moderate H_2O_2 production in cells from the first and second subpopulations (Table 2).

These results clearly show that conversion from the first to the second subpopulation is accompanied by a marked increase in membrane expression of Le^X, sLe^X, and β2-integrin antigens in H_2O_2 production as well as in the cell content of polymerized actin. Le^X expression in the second subpopulation (before and after stimulation) was as strong as that found on granulocytes in resting and stimulated conditions (data not shown). As CD14 antigen up-regulation has been reported on stimulated neutrophils,[35,36] it was important to ensure that CD14^{high} cells were not neutrophils whose CD14 expression was up-regulated in our experimental conditions. Dual immunofluorescence staining with FITC-anti-HLA-DR and anti-CD14 monoclonal antibodies was therefore performed in the presence and absence of LPS. CD14^{high} cells were

Table 2. Effect of LPS Stimulation on the Percentages and Features of Two CD14^{high} Subpopulations*

Features of monocytes	First subpopulation		Second subpopulation	
	PBS	LPS	PBS	LPS
Percentage	89 ± 03	54 ± 03	11 ± 03	46 ± 02
Anti-Le^x mAb†	12 ± 02 (10 ± 02)‡	22 ± 04 (11 ± 02)‡	940 ± 128 (152 ± 46)‡	1669 ± 197 (798 ± 96)‡
Anti-sLe^x mAb†	297 ± 137 (261 ± 65)‡	229 ± 116 (110 ± 25)‡	1282 ± 151 (161 ± 44)‡	865 ± 66 (369 ± 36)‡
DCF†	23 ± 05	38 ± 05	74 ± 12	146 ± 27
Polymerized actin†	49 ± 04	85 ± 17	187 ± 39	267 ± 18
Anti-CD11b mAb†	43 ± 03	187 ± 10	66 ± 38	560 ± 38
Anti-CD18 mAb†	40 ± 03	127 ± 03	69 ± 31	304 ± 22

*Whole blood was incubated with PBS or LPS (5 μg/ml) for 30 minutes. Samples were then treated as in Table 1.
†See legend of Table 1.
‡Total expression of Le^x and sLe^x expressed by each subpopulation ($\times 10^{-2}$) (ie its percentage multiplied by its mean fluorescence intensity) is given in brackets.
Values are means ± SEM (n=10).

all positive for HLA-DR and could therefore be considered as monocytic cells.[37] Moreover, experiments on isolated granulocytes CD14-positive showed that isolated neutrophils did not express CD14 antigen as strongly as monocytes. In addition, whole blood was incubated with LPS (5 μg/ml for 30 minutes) and the second subpopulation of CD14^{high}/CD15$^+$ cells were then sorted using a FACSsort apparatus and collected for cytospin preparation. Microscopic examination showed cells of the monocyte lineage. Finally, using propidium iodide, we demonstrated that CD14^{high}/CD15$^+$ cells were not aggregates of cells such as monocyte-neutrophils. All of these results showed that the two subpopulations represented two activation states of the same monocyte population.

Origin of Increased Membrane Expression of Le^X and sLe^X Antigens

As shown in Tables 1 and 2, monocyte stimulation triggered abundant conversion from the first to the second subpopulation, and this conversion involved increased membrane expression of both Le^X and sLe^X antigens.

One possible explanation for the strong Le^X and sLe^X antigens expression by the second subpopulation was that monocyte activation by LPS, while converting the first subpopulation into the second subpopulation, induced membrane translocation of intracellular molecules of Le^X or sLe^X, as probably occurred with CD11b and CD18. We thus performed simultaneous detection of membrane and intracellular Le^X or sLe^X after methanol permeabilization. The absolute fluorescence intensities obtained in these conditions could not be compared with those obtained at the cell surface only as methanol modifies fluorescence emission. However, the ratio of fluorescence intensities between the second and first subpopulations could be compared after permeabilization (total cellular expression). With regards to sLe^X, the ratio was 1:1 after permeabilization, indicating that the total sLe^X content was the same in the first and second subpopulations. Therefore, an intracellular pool of sLe^X was translocated to the membrane during conversion of the first to the second subpopulation. While Le^X was only expressed at the cell surface of the second subpopulation, permeabilization revealed its presence in the first subpopulation with a ratio of 10 between the second and first subpopulations. Membrane Le^X expression, observed in the second subpopulation before stimulation, increased as described above after LPS stimulation, much more than would be expected from its intracellular pool. This up-regulation could be related to the translocation of Le^X to the membrane in conjunction with increased sialidase activity[38] acting on a translocated intracellular pool of sLe^X. Le^X expression after incubation with PBS or LPS was not significantly modified by cycloheximide (data not shown), suggesting that Le^X up-regulation did not necessitate *de novo* protein synthesis.

We then investigated whether the degranulation process after stimulation contributed to the conversion of the first to the second subpopulation. Cytochalasin B (5 μg/ml), which promotes stimulus-induced degranulation,[39–41] increased the percentage of cells in the second subpopulation (Table 3); in particular, after LPS stimulation, the second subpopulation of CD14^{high} cells expanded from 35% (in the absence of cytochalasin B) to 63% (in the presence of cytochalasin B). In contrast, pentoxifylline (10 μmol/L), which inhibits degranulation,[42] strongly reduced the percentage of cells in the second subpopulation (Table 3). Similar results were obtained with colchicine (10 μmol/L) and ethanol (1%). These results support the involvement of degranulation in conversion of the first to the second subpopulation.

Table 3. Effect of Cytochalasin B and Pentoxifylline on the Percentage of the Second Subpopulation of CD14^{high} cells

Addition	PBS	Cytochalasin B	Pentoxifylline
PBS	10 ± 3	31 ± 6	4 ± 1
LPS	35 ± 5	63 ± 5	6 ± 2

The second subpopulation was defined as the subpopulation exhibiting an increase in size anf forward scatter. PBS, cytochalasin B (5 μg/ml), or pentoxifylline (10 mmol/L) was added to whole blood 5 minutes before the addition of PBS or LPS.
Values are means ± SEM (n = 3).

Table 4. Effect of Preincubation with LPS on the Monocyte Respiratory Burst Induced by fMLP in Whole Blood

Experimental conditions	First subpopulation		Second subpopulation		
	MFI	SI	MFI	SI	Percentage
PBS + PBS	23 ± 03	1	74 ± 12	1	11 ± 3
PBS + fMLP	31 ± 04*	1.39 ± 0.07*	96 ± 16*	1.33 ± 0.14*	20 ± 4*
LPS + PBS	38 ± 05*	1.78 ± 0.15*	146 ± 27*	2.12 ± 0.38*	46 ± 2*
LPS + fMLP	57 ± 17*	2.89 ± 0.34*	444 ± 68**	6.75 ± 1.20**	56 ± 4*

After preincubation with DCFH-DA (100 μmol/L) for 15 minutes at 37°C, whole blood was pretreated with PBS or LPS (5 μg/ml) for 30 minutes and then with PBS or fMLP at 10^{-6} mol/L for 5 minutes. Anti-CD14 antibody was used to identify the monocyte population. The mean fluorescence intensity of dichlorofluorescein was recorded as described in Materials and Methods.

Values are means ± SEM (n = 10).

*Significantly different from control incubated with PBS alone.

**Significantly different from control incubated with PBS alone and from the sum of MFI of the samples incubated with fMLP or LPS alone (associated each with PBS).

SI, stimulation index.

Increased H_2O_2 Production by the Second Subpopulation of $CD14^{high}$ Cells in Whole Blood

As shown in Table 4, fMLP (10^{-6} mol/L for 5 minutes) or LPS (5 μg/ml for 30 minutes) added separately to whole blood induced a significant but moderate H_2O_2 production by $CD14^+$ cells from the first subpopulation (stimulation index = 1.39 ± 0.07 and 1.78 ± 0.15 for fMLP and LPS, respectively). In these conditions of stimulation, the stimulation index from the first and the second subpopulation had the same order of magnitude. However, preincubation of whole blood with 5 μg/ml LPS for 30 minutes, followed by treatment with 10^{-6} mol/L fMLP for 5 minutes, induced a marked increase in H_2O_2 production by the second subpopulation (stimulation index = 6.75 ± 1.20), which was significantly (eightfold) higher than that of the first subpopulation (Table 4). Interestingly, fMLP-induced H_2O_2 production (second subpopulation) by LPS-primed monocytes was significantly and markedly higher than that generated individually by fMLP and LPS (Table 4). H_2O_2 production was of same order as that of neutrophils in the same priming conditions (data not shown).

LPS has been reported to interact with a number of specific cellular recognition proteins at the monocyte surface, especially the CD14 molecule. To analyze whether LPS primed monocytes through an interaction with CD14 alone or in concert with other signaling proteins expressing carbohydrate structures such as Le^X or sLe^X, whole blood was treated with monoclonal anti-CD15 and anti-sLe^X antibodies for 5 minutes before LPS treatment. Anti-CD11b, anti-L-selectin, and anti-PECAM were also analyzed. Anti-CD14 was used as a positive control and an IgM control isotype with irrelevant specificity was matched with the anti-CD15 and anti-sLe^X antibodies. Treatment with monoclonal anti-CD14 did not modify DCFH oxidation in samples incubated with PBS or fMLP alone (data not shown). In contrast and as expected,[43] incubation with anti-CD14 antibody before LPS priming strongly reduced H_2O_2 production by $CD14^{high}$ cells from the two subpopulations relative to samples treated with LPS alone (Figure 2). Unexpectedly, similar results were obtained after preincubation with anti-CD15 and anti-sLe^X antibodies, whereas preincubation with a mouse IgM control isotype (Figure 2) as well as with antibodies against other molecules at the monocyte surface (anti-PECAM, anti-CD11b, and anti-L-selectin antibodies) did not modify H_2O_2 production by LPS-treated monocytes (data not shown). Furthermore, anti-CD14, anti-CD15, and anti-sLe^X monoclonal antibodies decreased H_2O_2 production by both subpopulations after fMLP stimulation of LPS-primed monocytes (LPS pretreatment followed by fMLP stimulation); the fall was larger in the second subpopulation (Figure 3). We checked that the addition of anti-CD-15, anti-sLe^X, and anti-CD14 antibodies did not modify the balance between the two subpopulations. The percentages of cells in the second subpopulation were 54.1 ± 3.2, 54.7 ± 3.8, 54.3 ± 3.5, and 54.0 ± 3.5 after incubation with PBS, anti-CD15, anti-sLe^X, and anti-CD14, respectively. In addition, no alteration in scatter parameters or viability (propidium iodide staining) was observed after incubation with the antibodies.

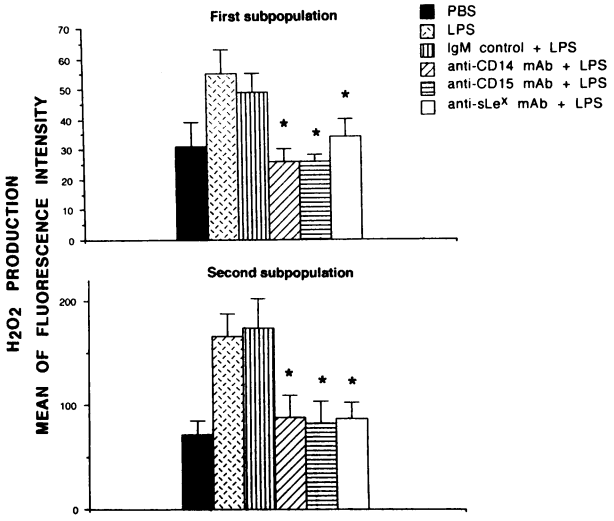

Figure 2. Effect of anti-CD14, anti-CD15, and anti-sLe^X mAbs on the LPS-stimulated monocyte oxidative burst. Whole blood was incubated with mAbs at 37°C for 5 minutes before stimulation with LPS. The mean of fluorescence intensity of DCF in the first and second subpopulation was recorded as described in Materials and Methods. Values obtained with isotypic control (IgG2b) of anti-CD14 mAb and with anti-L-selectin, anti-CD11b, and anti-PECAM mAbs were not significantly different from that of IgM isotypic control of anti-CD15 mAb and anti-sLe^X mAb. Values are means ± SEM (n = 3). *Significantly different from control incubated with LPS alone ($P < 0.05$).

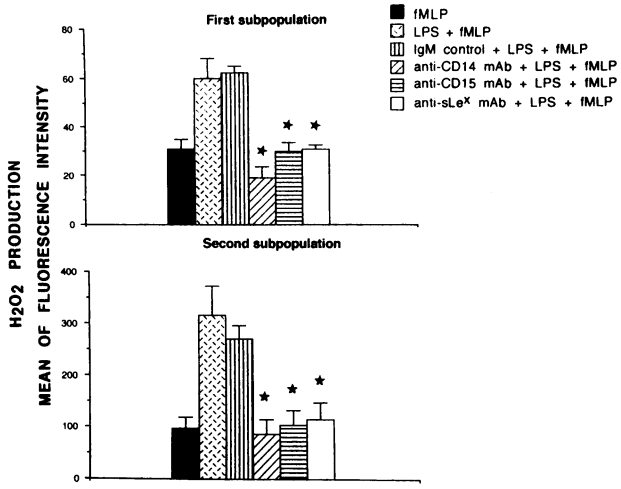

Figure 3. Inhibition by anti-CD15 and anti-sLe^X antibodies of LPS-primed oxidative burst in response to fMLP. Whole blood was incubated with mAbs at 37°C for 5 minutes before treatment with LPS and stimulation with fMLP. The mean of fluorescence intensity of DCF in the first and second subpopulation was recorded as described in Materials and Methods. Values obtained with isotypic control (IgG2b) of anti-CD14 mAb and with anti-L-selectin, anti-CD11b, and anti-PECAM mAbs were not significantly different from that of IgM isotypic control of CD15 mAb and sLe^X mAb. Values are means ± SEM (n = 3). *Significantly different from control incubated with LPS + fMLP ($P <$ 0.05).

In contrast, addition of anti-CD14, anti-CD15, or anti-sLe^X to monocytes after their priming with LPS and before fMLP addition did not reduce the increased H_2O_2 production by the primed monocytes (Figure 4). These results suggest that Le^X and sLe^X are involved in the priming itself and not in the NADPH oxidase activity of already primed monocytes. To analyze whether the inhibition of LPS-priming by anti-Le^X or anti-sLe^X was due to their interference with LPS binding, the binding of labeled LPS was measured in their presence and absence. FITC-LPS binding was similar after preincubation with anti-Le^X, anti-sLe^X, IgM control isotype, or anti-PECAM antibody, whereas it was significantly inhibited in presence of anti-CD14, as expected (Figure 5).

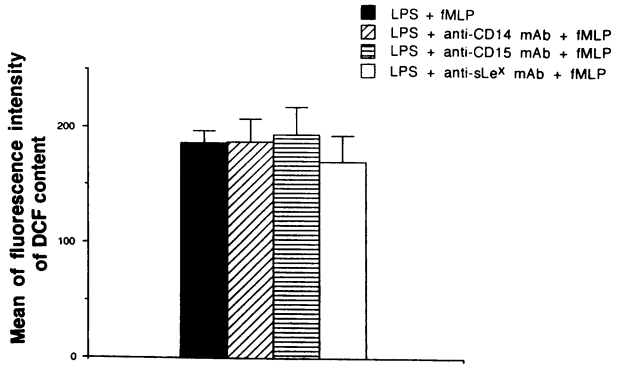

Figure 4. Effect of anti-CD14, anti-CD15, and anti-sLe^X mAbs when added after LPS and before fMLP on monocyte oxidative burst of the second subpopulation. After preincubation with DCFH-DA (100 μmol/l) for 15 minutes at 37°C, whole blood was pretreated with LPS (5 μg/ml) for 30 minutes; mAbs were then added for 5 minutes before stimulation with fMLP. The mean fluorescence intensity of DCF was recorded as described in Materials and Methods. Values are means ± SEM (n = 3). The graphs depict CD14^{high} cells from the second subpopulation. Similar results were observed with cells from the first subpopulation.

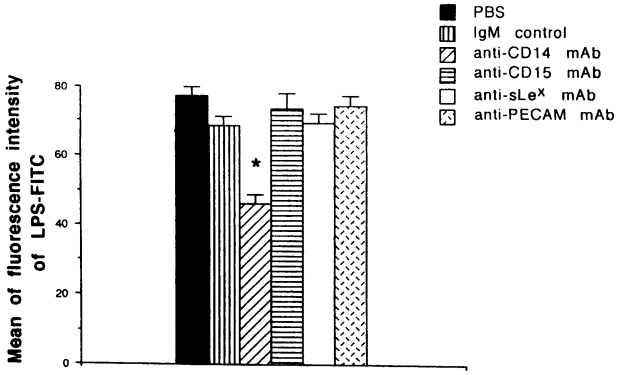

Figure 5. Effect of anti-CD14, anti-CD15, anti-sLe^X, and anti-PECAM mAbs on LPS binding at the surface of CD14^{high} cells from the second subpopulation. Whole blood was incubated with mAbs at 37°C for 5 minutes before treatment with LPS-FITC for 30 minutes. The mean fluorescence intensity of LPS-FITC was recorded as described in Materials and Methods. Values are means ± SEM (n = 3). *Significantly different from control incubated with LPS alone. The graphs depict CD14^{high} cells from the second subpopulation. Similar results were observed with cells from the first subpopulation.

Discussion

In addition to classically recognized monocytes, we identified a subpopulation of whole-blood monocytes (representing 8% of all monocytes) with higher scatter parameter and strong CD11b, CD18, Lewis-X (Le^X, CD15, 3-α-fucosyl-*N*-acetyl-lactosamine), and sialyl-Lewis-X (sLe^X) antigen expression at the cell surface as well as an increased polymerized actin content. The size of this minor subpopulation increased at the expense of the major subpopulation after stimulation with LPS. The unexpected increase in Le^X and sLe^X antigen expression during this conversion was due, in large part, to translocation of these antigens from intracellular pools, probably associated for the increased Le^X expression to partial desialylation of sLe^X. Moreover, the monocyte subpopulation with high scatter parameters and expressing high levels of CD15 and sLe^X antigen produced large amounts of H_2O_2 in response to fMLP after LPS priming. Primed H_2O_2 production was inhibited by the addition of anti-CD14, anti-CD15, or anti-sLe^X monoclonal antibody before LPS. This was not the case when the antibodies were added after LPS and before fMLP, suggesting that Le^X and sLe^X regulate LPS priming of blood monocytes.

The cells identified by flow cytometric analysis with a phycoerythrin-CD14 monoclonal antibody were monocytes, as they were characterized by strong expression of CD14 antigen on the fluorescence histogram. Neutrophils also express CD14 but at a lower level[36,44] and were excluded from analysis on this basis. Consequently, this analysis did not include the monocyte subpopulation expressing low CD14 antigen and high CD16 antigen levels described by Ziegler et al.[45] As surface expression of CD14 antigens on neutrophils has been reported to be up-regulated by LPS[36] and formyl peptides[46] and as Le^X is considered a marker of neutrophils,[19,20] we further studied the subpopulation of CD14^{high}/CD15$^+$ cells to confirm that they were monocytes. This was clearly the case, as 1) HLA-DR antigen, which is expressed by monocytes and not by neutrophils in our conditions of

stimulation,[37] was detected at the surface of $CD14^{high}$/$CD15^{+}$ cells; 2) isolated neutrophils, which are $CD15^{+}$, did not express CD14 antigen as strongly as isolated monocytes after LPS stimulation; 3) sorting of $CD14^{high}$/$CD15^{+}$ cells and microscopic examination confirmed that they belonged to the monocyte lineage; and 4) $CD14^{high}/CD15^{+}$ cells were not aggregates, particularly of the monocyte-neutrophil type.

The monocyte subpopulation with high scatter parameters shared certain characteristics (high scatter parameters, high CD11b, and actin polymerization, as well as high production of reactive oxygen species) with the subpopulation previously identified by Owen et al[8,9] in monocytes isolated by countercurrent centrifugal elutriation. Working on whole blood with minimal uncontrolled stimulation, we found that the subpopulation represented 8% of $CD14^{high}$ cells, compared with 20 to 30% in the study by Owen. The smaller size of our subpopulation could be attributed to the absence of isolation procedures. Indeed, after stimulation of whole blood with LPS, the increase in the percentage of cells in the subpopulation with high scatter parameters was matched by a parallel reduction in $CD14^{high}$ cells that did not express these characteristics. Our results thus suggest that the second subpopulation described here is composed of activated monocytes rather than representing a distinct subpopulation originating in the bone marrow; in fact, more potent stimulation than that associated with blood collection, such as that induced by LPS, clearly converted monocytes of the first subpopulation into activated monocytes with strong expression of CD11b and CD18 and a high polymerized actin content as well as a high oxidative burst response, which are characteristics similar to those of the second subpopulation.

Le^{X} antigen (CD15) is strongly expressed on neutrophils of which it is a major phenotypic marker.[19,20] In contrast, anti-CD15 monoclonal antibodies have been reported to react less strongly with monocytes: monocytic differentiation of HL60 cells was associated with a decrease in Le^{X} antigen expression.[18] sLe^{X} antigen is expressed on both monocytes and neutrophils. Our study of whole blood confirms the absence of Le^{X} expression by the majority (92%) of $CD14^{high}$ monocytes and shows the existence of some monocytes (8%) strongly expressing Le^{X}. In addition, this second subpopulation expressed higher levels of sLe^{X} than the first. The fact that LPS stimulation significantly increased the percentage of the second subpopulation suggested the existence of an intracellular pool of Le^{X} and sLe^{X} that could be expressed on the membrane after stimulation. This was supported by the recognition of these pools in permeabilized monocytes and by the effect of cytochalasin B, a degranulating agent, which increased the percentage of the second $CD14^{high}$ subpopulation. In contrast, agents that inhibit degranulation, such as pentoxyfilline, ethanol, and colchicine, reduced the percentage of the second $CD14^{high}$ subpopulation. However, whereas permeabilization revealed intracellular Le^{X} in the first subpopulation, the amount was too low to account for its membrane expression after LPS activation. This led us to suspect that expression of Le^{X} during conversion of the first to the second subpopulation resulted both from its intracellular pool and from desialylation, as it has been reported that monocyte sialidase can be activated after monocyte stimulation and can convert sLe^{X} into Le^{X}.[38,47] Additional studies are needed to determine whether other mechanisms such as *de novo* generation of these carbohydrate structures are associated with those analyzed here. LPS-induced monocyte expression of Le^{X} was not significantly modified by cycloheximide (data not shown), meaning that its up-regulation was probably not related to *de novo* protein synthesis.

Le^{X} and sLe^{X} are carbohydrate ligands recognized by endothelial selectins and involved in the initial interactions of circulating monocytes with the endothelium in a process known as rolling adhesion.[12,13,14] At the monocyte surface, sLe^{X} is the specific carbohydrate recognized by endothelial selectins.[14] Therefore, the stronger expression of sLe^{X} antigen at the $CD14^{high}/CD15^{+}$ cell surface may play a key role in the initial interactions of this monocyte subpopulation with the endothelium.

H_2O_2 production by unstimulated cells and LPS- or fMLP-stimulated cells was significantly higher in the second subpopulation ($CD14^{high}/CD15^{+}$ cells) than in the first subpopulation. There have been conflicting reports on the direct effect of LPS on the oxidative burst of monocytes isolated from their blood environment; some authors have reported that LPS has a triggering effect,[43,48] whereas others found no such effect.[27,49] These discrepancies might be related to isolation procedures that have been shown to alter surface antigen expression and thus might interfere with stimulation pathways generating the oxidative burst. We clearly observed that LPS had a priming effect on H_2O_2 production by $CD14^{high}$/$CD15^{+}$ cells in response to formyl peptides. Indeed, after preincubation of whole blood with LPS for 30 minutes followed by treatment with fMLP for 5 minutes, H_2O_2 production was about twofold higher than the sum of that obtained with each stimulus alone. This priming phenomenon was only observed with $CD14^{high}/CD15^{+}$ cells of the second subpopulation. In contrast to the generally accepted idea that monocytes produce less reactive oxygen species than neutrophils,[3] the degree of H_2O_2 production by $CD14^{high}/CD15^{+}$ cells was similar to that obtained with primed neutrophils in the same conditions (ie, whole blood; data not shown).

Previous studies have shown that CD14 mediates H_2O_2 production by monocytes stimulated with LPS[42] or primed with IFNγ.[50,51] Our finding that incubation of whole blood with anti-CD14 monoclonal antibody before stimulation with LPS strongly reduced H_2O_2 production in response to LPS, and LPS + fMLP is in keeping with these reports. However, CD14 is a glycosylphosphatidylinositol-linked protein that lacks a cytoplasmic domain and any known signal transduction sequence. Therefore, it could act in concert with other signaling proteins. Besides the CD14 molecule itself, the following structures serve as binding sites and/or receptors for LPS on human monocytes: the CD11b/CD18 complex, an 80-kd molecule of human leukocytes, and a lectin-like monocyte membrane molecule.[52] We clearly demonstrated that incubation of whole blood with anti-CD15 and anti-sLe^{X}

monoclonal antibodies strongly decreased LPS-induced H_2O_2 production and especially LPS priming to fMLP-induced oxidative burst, whereas this was not the case with anti-CD11b (Figure 2 and 3). The binding of anti-CD15 and anti-sLe^X antibodies to the cell surface may therefore block either LPS binding or a signal transduction step triggered by LPS, pointing to the involvement of these carbohydrate structures in the molecular events leading to the oxidative burst triggered by LPS. LPS binding measured by flow cytometry was not altered in our hands. Stimulated monocytes expressing Le^X may behave like neutrophils as previous studies have indicated that antibodies against CD15 antigen specifically inhibit the neutrophil oxidative burst in response to serum-opsonized yeast,[32,53] phorbol myristate acetate,[31,32,53] and formyl peptides.[31] Our results additionally show that anti-CD15 and anti-sLe^X antibodies inhibit LPS priming when added before LPS and not fMLP stimulation, as they had no effect when added before fMLP but after LPS.

In conclusion, we found a highly reactive and inducible subpopulation of monocytes in whole blood that share phenotypic (high Lewis-X expression) and functional characteristics with neutrophils (high LPS-primed ROS production). Furthermore, as both anti-Lewis-X and anti-sLe^X monoclonal antibodies inhibited the large amounts of H_2O_2 produced after LPS priming in response to fMLP, our results point to the involvement of these carbohydrate structures in LPS priming of the monocyte oxidative burst. This subpopulation of monocytes might play an important role in host defenses and inflammatory responses through strong production of ROS.

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