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CD14 and CD11b Mediate Serum-Independent Binding to Human Monocytes of an Acylpolygalactoside Isolated from *Klebsiella pneumoniae*

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A water-soluble acylpolygalactosyl (APG) of 34 kDa was obtained from the *Klebsiella pneumoniae* membrane by alkaline hydrolysis and delipidation. APG comprises a poly(1,3)galactose chain, a core, and a lipid moiety made of a glucosamine disaccharide with two N-linked βOH-myristates. The monocyte binding sites for APG were investigated by flow cytometry. Biotin-labelled APG (Biot-APG) bound to monocytes at 4°C in the absence of serum, calcium, and magnesium. The binding was dose dependent, saturable, and displaced by unlabelled APG. Neither the polysaccharide chain present in APG-related molecules nor the PP_i group or additional ester-linked myristates and palmitates were required for APG binding. The role of CD11b and CD14 was demonstrated by competitive inhibition with monoclonal antibodies and by the uptake of APG by these solubilized proteins. APG was rapidly internalized into monocytes at 37°C while CD14 and CD11b/CD18 molecules were partially down-modulated. Lipopolysaccharides (LPS) from the same *K. pneumoniae* strain and from *Escherichia coli* and *Salmonella minnesota* partially competed for Biot-APG binding in the absence but not in the presence of serum. When altered by alkaline hydrolysis, those LPS became strong competitors for APG binding. It was concluded that alkaline hydrolysis of the *K. pneumoniae* membrane yielded molecules structurally related to LPS which bind to LPS membrane receptors in the absence of serum.

Mononuclear phagocytes (monocyte/macrophages) represent the principal target for the lipopolysaccharides (LPS/endotoxin) of Gram-negative bacteria. LPS triggers a wide range of cellular responses, including the synthesis and release of a variety of inflammatory mediators (e.g., tumor necrosis factor alpha [TNF-α], interleukin 1 [IL-1], IL-6, IL-8, and prostaglandins) which contribute to host defense mechanisms but may induce major immunopathological disorders when produced in excess. The triggering of cellular responses is initiated by the binding of LPS to the cell surface which precedes its internalization (2, 18, 19, 27). Over the last few years, major efforts have been devoted to the characterization of cell membrane structures which may function as LPS receptors. The role of the integrin CD11b/CD18 has been reported (42, 43) as well as that of CD14 (45). However, LPS binding to and cell activation by CD14 were initially demonstrated in the presence of LPS-binding protein contained in normal serum (30, 35, 43). LPS may also bind to a p73 protein characterized in mouse and human cells (12, 22, 24), to the low-density lipoprotein "scavenger receptor" (13), and to the recently characterized bactericidal/permeability-increasing protein on the surface of polymorphonuclear leukocytes (38).

Identification and characterization of LPS-binding molecules at the cell surface are particularly difficult because LPS are highly hydrophobic and tend to form micelles in water. Furthermore, distinct regions of the LPS molecule may be involved in attachment to cells and triggering of cellular responses. We recently had the opportunity to investigate the structure and activity of several acylpolygalactoside molecules prepared by alkaline hydrolysis from the membrane of *Klebsiella pneumoniae* (Institut Pasteur I-145). These compounds possess the overall structure of an LPS molecule with a long poly(1,3)D-galactoside chain, a core region containing ketodeoxyoctonic acids (KDO) and heptoses, and a lipidic end made of two phosphoglucosamines, each bound to one N-linked βOH-myristate (Fig. 1).

Depending on the conditions of alkaline hydrolysis, two 34-kDa derivatives were obtained, acylpoly(1,3)galactoside (APG) and APG containing ester-linked fatty acids (EFA-APG), which differ by the acylation of their lipid region. These modified LPS are highly soluble in water and therefore permit further investigation of the structure-activity relationship within LPS molecules. We previously showed that those modified LPS could bind to human monocytes and to murine lymphoid cells irrespectively of the type of acylation of their lipid region, while the triggering of cellular activation was achieved only by EFA-APG, which differs from APG by the presence of two additional ester-linked fatty acids (14–17, 21). In this study, we used these different water-soluble altered LPS to further investigate the contribution of CD11b/CD18 and CD14 to the binding of these molecules in the absence of serum and to assess the region(s) of APG involved in the binding to monocytes.

MATERIALS AND METHODS

APG derivatives and LPS. The 34-kDa derivatives APG and EFA-APG were obtained from the *K. pneumoniae* membrane extract by two alkaline hydrolysis procedures and preparative gel chromatography as described elsewhere (15, 17). Briefly, the first procedure (mild hydrolysis with 0.1 N NaOH for 24 h

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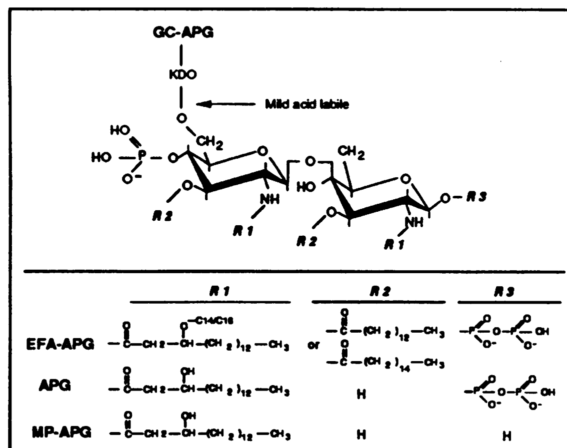

FIG. 1. Structure of the lipid part of APG molecules.

at 22°C) yielded a homogeneous 34-kDa fraction containing EFA-APG. The second procedure (drastic alkaline hydrolysis with 0.5 N NaOH for 1 h at 56°C, performed twice before and after a delipidation step) yielded the 34-kDa fraction APG. GC-APG, a 28-kDa molecule derived from APG by acid hydrolysis (acetic acid [1%, vol/vol], 45 min, 100°C) contains mostly isolated poly(1,3)galactose chains.

Monophosphoryl APG (MP-APG) was obtained by heating an APG solution at 80°C for 16 h. LPS from the same strain of *K. pneumoniae* (Kp-LPS) was extracted by the phenol-water method (39), and LPS from the Re strain of *Salmonella minnesota* (Re-LPS) and *Escherichia coli* O111:B4 (Ec-LPS) were from Sigma (St. Louis, Mo.). Kp-LPS and Ec-LPS submitted to alkaline hydrolysis (0.5 N NaOH, 1 h, 56°C) were referred to as Kp-LPS(NaOH) and Ec-LPS(NaOH). The detailed analytical compositions of the APG derivatives and Kp-LPS have been described in previous reports (15, 17). The common structure of the 34-kDa derivatives is a linear polysaccharide chain composed exclusively of poly(1,3)D-galactose linked to a core-like region which contains two KDO molecules. The lipid part of APG comprises a glucosamine disaccharide with a PP_i at C-1 and a phosphate at C-4′. Each glucosamine is linked by amide bond to one βOH-myristate (Fig. 1). EFA-APG differs from APG by the presence of two additional ester-linked myristic and palmitic acids. MP-APG is an APG dephosphorylated at C-1 (Fig. 1).

Labelling of APG with biotin and FITC. Succinimidyl-biotin (Sepracor IBV, Villeneuve la Garenne, France) at 5 mg/ml in *N,N*-dimethylformamide was used for labelling of APG. The biotin solution (30 μl) was added to 10 mg of APG in 2 ml of sodium carbonate buffer (0.2 M, pH 8.8), and the reaction was allowed to proceed for 15 min at room temperature on a rotary shaker. The biotinylation was stopped by addition of 40 μl of NH_4Cl (1 M, pH 6), and biotin-labelled APG (Biot-APG) was dialyzed for 3 days against NaCl (0.15 M, 4°C) and frozen until use. APG was labelled with fluorescein isothiocyanate (FITC) (isomer I; Sigma) in sodium carbonate buffer as described elsewhere (14, 17). As demonstrated for FITC-APG (14), coupling of the molecule with biotin did not alter the functional properties of APG as assessed by controlling for the capacity of Biot-APG to stimulate monocyte oxidative bursts.

PBMC. Heparinized blood samples of healthy volunteers were fractionated by centrifugation (20 min, 400 × *g*, 20°C) on a cushion of Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway). Peripheral blood mononuclear cells (PBMC) were harvested from the Ficoll-plasma interface and washed twice in Hank's balanced salt solution (HBSS; Vietech, St. Bonnet de Mure, France).

MAbs. The following anti-CD11/CD18 monoclonal antibodies (MAbs) were used: anti-α chain of LFA-1 (CD11a), IOT16 (Immunotech, Luminy, France), BL5 (prepared by J. Brochier in our laboratory), and AFOL1 (Pasteur/Mérieux, Sérums et Vaccins, Lyon, France); anti-α chain of CR3 (CD11b), Leu15 (Becton Dickinson, Mountain View, Calif.), IOMb1 (Immunotech), and M1/70 (hybridoma from American Tissue Culture Collection, Rockville, Md.); anti-α chain of p150-95 (CD11c), LeuM5 (Becton Dickinson), and MAb IOMc (Immunotech); and anti-common β chain (CD18), 60.3 (Dako SA, Trappes, France). Anti-CD14 MAbs were IOM2 (Immunotech), LeuM3 (Becton Dickinson), and BA-8 (a gift from J. Wijdenes, Besançon, France), and anti-RFcγIII (CD11c) MAb was 3G8 (Immunotech). Anti-HLA-DR MAb was BL2 (from J. Brochier), and anti-HLA-class I MAb was W6/32 (Dako). Noncommercially available MAbs were purified from ascites on protein A-Sepharose (Pharmacia, Uppsala, Sweden). The nonoverlapping character of the epitopes recognized by MAbs on CD11a, CD11b, CD11c, CD18, and CD14 was established in competitive binding experiments with biotinylated AFOL1, M1/70, IOMc, BL5, and IOM2 and unlabelled competing MAbs. A γ1 isotype MAb (8545 A), anti-secretory component (37), was used as irrelevant control antibody.

Binding studies. Binding studies were performed at 4°C in serum-free medium. Suspensions of 10^6 PBMC were washed with binding buffer (HBSS with 0.1% gelatin and 0.1% sodium azide), and the pellet was stained in binding buffer by indirect immunofluorescence using Biot-APG (20 μl per pellet) for 30 min followed by subsequent incubation with streptavidin-phycoerythrin (Caltag Laboratories, San Francisco, Calif.) (1:20, 20 μl) and two washes in ice-cold binding buffer. After each step, unbound reagents were removed by two washes in binding buffer and cells were fixed with 1% formaldehyde in binding buffer. Binding of Biot-APG was measured in the absence of competitor (total binding) and in the presence of a 10-fold excess of unlabelled APG to determine the nonspecific binding. All results were expressed as net mean fluorescence intensity (ΔMFI) after subtraction of the background MFI (cells plus buffer plus streptavidin-phycoerythrin used as a control). Specific binding was calculated by subtracting nonspecific binding from total binding.

To analyze the binding of mouse MAbs to monocyte membrane molecules, MAb-labelled cells were further incubated for 30 min with FITC-conjugated $F(ab')_2$ of goat anti-mouse immunoglobulin (Tago Immunologicals, Burlingame, Calif.) and then washed twice and fixed. For competitive inhibition by MAbs, cells were first incubated with a saturating concentration of MAb before addition of Biot-APG. The role of divalent cations was studied by using HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer instead of HBSS in the presence of either EDTA (10 mM) or $CaCl_2$ and/or $MgCl_2$ (0.5 mM).

Enzymatic treatment by PI-PLC. To deplete monocytes of CD14, PBMC (8 × 10^6 cells per ml) were incubated for 2 h at 37°C in RPMI 1640 (Sigma) and in the presence of various concentrations of phosphatidyl inositol phospholipase C (PI-PLC) from *Bacillus cereus* (Sigma). After incubation, cells were washed twice in binding buffer and labelled with Biot-APG as described above. LeuM3 MAb coupled with FITC (Becton Dickinson) was used to evaluate the efficiency of surface CD14 removal.

Flow cytometry. Cell suspensions were analyzed by using a

FACScan cytofluorometer (Becton Dickinson) which was equipped with an argon ion laser operating at 488 nm and 250-mV light output. To analyze the monocytes separately, side-scatter parameters were used to apply optimal computerized gating (2,000 to 5,000 events). The relative fluorescence intensity of each subset was recorded as single-parameter histograms (log scale, 256 channels, 48 channels per log decade). Fluorescence measurements were converted from logarithmic to linear by using a logarithmic-linear calibration factor, and the results were expressed as ΔMFI after subtraction of the background MFI (control, cells plus binding buffer instead of the ligands). Specific binding was calculated by subtracting the ΔMFIs corresponding to the Biot-APG bound to the cells in presence of excess unlabelled ligand from those corresponding to Biot-APG bound in the absence of unlabelled APG.

Confocal laser microscopy. Monocytes were separated from PBMC by adherence to plastic in RPMI 1640 containing 10% heat-inactivated endotoxin-free fetal calf serum (FCS) (Seromed, Noisy-le-Grand, France) for 1 h and recovered by using a rubber policeman. Monocytes were incubated with FITC-APG (10 μM) for various times at 37°C in RPMI 1640 with 2% FCS, and control samples were incubated at 4°C in medium containing 0.1% sodium azide. Intracellular fluorescence was observed by confocal laser microscopy applied to cells stained and mounted in Tris-glycerol-saline buffer; optical slices were generated in a Zeiss confocal laser scanning microscope (model LSM 10).

Extraction of cell membrane proteins. Membrane proteins from a pellet of 5×10^7 cells were extracted for 15 min on ice in 1 ml of 10 mM Tris acetate (pH 8.0) containing 0.5% Nonidet P-40, phenylmethylsulfonyl fluoride (0.35 mg/ml), iodoacetamide (9.25 mg/ml), and pepstatine A (1 μg/ml). Extracts were cleared of nuclei and cell debris by centrifugation at 48,500 × *g* and 4°C for 45 min. Supernatants were collected and kept at −70°C until use.

Solid-phase binding assay. To examine the interaction of Biot-APG with solubilized membrane proteins, a solid-phase binding assay was developed. Wells of a flat-bottomed 96-well polystyrene microplate (Costar, Cambridge, Mass.) were filled with 50 μl of 0.02 M sodium barbital buffer (pH 8.6) containing one of the MAbs at 10 μg/ml and incubated for 1 h at 37°C and then overnight at 4°C. Plates were washed three times with coating buffer, and the remaining antigen attachment sites were blocked by adding 100 μl of 0.5% gelatin in barbital buffer per well and incubating for 1 h at 37°C. After three washes with phosphate-buffered saline (PBS) containing 0.1% gelatin (PBS-Gel), duplicates of Nonidet P-40 extract diluted to 1/10 in PBS-Gel were added and left for 1 h at 37°C. After a washing, 50 μl of 5 μM Biot-APG in PBS-Gel per well was added and the mixture was left for 1 h at 37°C. After three washes, 50 μl of streptavidin-horseradish peroxidase complex (Amersham Int plc, Amersham, United Kingdom) was added and the mixture was incubated for 1 h at 37°C. The plate was then washed and 100 μl of substrate (0.08% *o*-phenylenediamine in citrate buffer [pH 5.5] containing 0.03% [vol/vol] H_2O_2) was added to each well. The enzymatic reaction was allowed to proceed for 15 min at room temperature and then quenched with 100 μl of 2 N H_2SO_4. The A_{492} was measured (reference, 620 nm) on a Multiscan plate reader (Titertek Labsystems, Les Ulis, France). Addition of buffer instead of Nonidet P-40 extract permitted controlling for nonspecific Biot-APG adsorption.

RESULTS

Specificity of the binding of APG to human monocytes. PBMC received increasing amounts of Biot-APG in the absence or presence of a 10-fold excess of unlabelled APG in serum-free buffer at 4°C. The binding of APG to monocytes as a function of ligand concentration is expressed as ΔMFIs in Fig. 2a. Specific binding of Biot-APG to monocytes was dose dependent and saturable. Saturation was achieved at 5 μM Biot-APG. Figure 2b shows that specific binding of Biot-APG occurred also in saline-HEPES buffer with or without 10 mM EDTA instead of HBSS, whereas the addition of Ca^{2+} and Mg^{2+} (0.5 mM) did not enhance the specific binding. Therefore, APG binding to monocytes was not dependent on the divalent cations Ca^{2+} and Mg^{2+}.

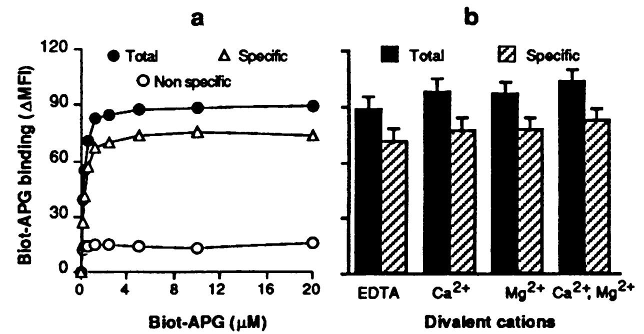

FIG. 2. (a) Dose-response and specificity of Biot-APG binding to human monocytes. Binding experiments and calculation of specific binding were performed as described in Materials and Methods. (b) Effect of extracellular Ca^{2+} and Mg^{2+} on specific binding. PBMC were washed twice in saline HEPES buffer, and binding of Biot-APG (5 μM, in the presence or absence of a 10-fold excess of unlabelled APG) in saline HEPES buffer supplemented with EDTA (10 mM) or with $CaCl_2$ (0.5 mM) and/or $MgCl_2$ (0.5 mM) was assessed. Data are means ± standard errors of the means of four experiments.

APG submolecular structures involved in the binding to monocytes. To localize the region of the APG molecule which contributes to the binding to the monocyte surface, we investigated the capacities of MP-APG and GC-APG to inhibit Biot-APG binding. Unlabelled MP-APG inhibited Biot-APG binding to monocytes as well as APG, indicating that interaction of APG with the cell membrane was not modified by the absence of the PP_i group at position C-1 (Fig. 3). Conversely, GC-APG did not inhibit Biot-APG binding, indicating that the polysaccharide chain of APG did not participate in the binding

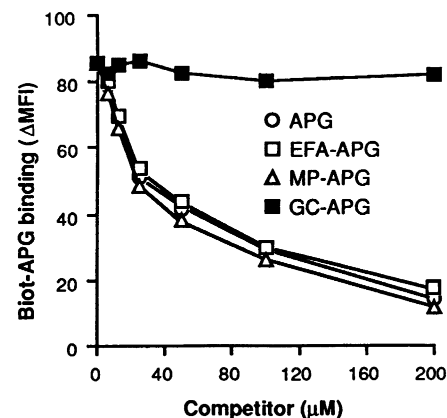

FIG. 3. Inhibition of binding of Biot-APG (5 μM final concentration) to monocytes by increasing amounts of unlabelled APG, EFA-APG, GC-APG, and MP-APG.

TABLE 1. Inhibition of the specific binding of Biot-APG to monocytes by MAbs[a]

MAb(s)	Membrane antigen(s)	% Inhibition[b]	MAb(s)	Membrane antigen(s)	% Inhibition
IOT16	CD11a	0 (0–0)	60.3	CD18	0 (0–0)
AFOL1	CD11a	2 (2–2)	IOM2	CD14	46 (33–53)
BL5	CD11a	3 (0–8)	BA-8	CD14	0 (0–0)
M1/70	CD11b	36 (27–41)	LeuM3	CD14	3 (3–3)
OKM1	CD11b	9 (8–18)	M1/70 + IOM2	CD11b + CD14	64 (56–72)
Leu15	CD11b	0 (0–1)	3G8	CD16 (FcγIIIR)	0 (0–0)
IOMb1	CD11b	1 (1–1)	W6.32	HLA class I	0 (0–0)
LeuM5	CD11c	12 (7–17)	BL2	HLA class II	4 (1–7)
IOMc	CD11c	0 (0–0)			

[a] Pellets of 10^6 PBMC were incubated for 30 min at 4°C in 20 μl of a saturating concentration of one of various MAbs and received Biot-APG (5 μM final concentration) in the presence or absence of a 10-fold excess of unlabelled APG. The cells were washed twice, and bound APG was revealed by a second incubation with streptavidin-phycoerythrin. Samples were processed by FACScan, and ΔMFI and specific binding were determined as described in Materials and Methods.

[b] Percent inhibition of specific binding of Biot-APG calculated as 100 × (1 − ΔMFI in the presence of antibody/ΔMFI in the absence of antibody). Values are means from four experiments (ranges are given in parentheses).

to cells. In addition, EFA-APG and APG displayed identical binding inhibition capacities (Fig. 3), showing that additional ester-linked fatty acids in the lipid part of APG did not enhance its binding to the cell membrane.

Specific binding of APG to monocytes is mediated by CD11b and CD14. The role of monocyte membrane antigens as binding sites for APG on monocytes was investigated by experiments with serum-free conditions at 4°C in the presence or absence of 10-fold excess unlabelled APG with cells preincubated for 30 min with a saturating concentration of one of various mouse or rat MAbs. Preincubation of cells with anti-HLA-class I or class II, anti-RFcγIII, anti-CD11a, anti-CD11c, or anti-β chain of CD11/CD18 integrins did not result in inhibition of specific binding of Biot-APG (Table 1). Conversely, significant inhibition was observed with M1/70 (anti-CD11b) and IOM2 (anti-CD14) MAbs (36 and 46%, respectively) but not with other MAbs against CD11b and CD14. Of note, the combination of anti-CD11b and anti-CD14 led to greater inhibition of APG binding (64%). By crossed-inhibition experiments, we observed that M1/70, Leu15, and IOMb1 do not recognize the same epitope on the CD11b molecule. The same conclusion was made for IOM2, BA8, and LeuM3 antibodies on the CD14 molecule (data not shown).

To confirm that CD11b and CD14 were involved in APG binding, we carried out the following experiment. Wells of microplates were coated with MAbs directed against CD11b or CD14 (IOMb1 and BA-8, respectively) or with anti-CD11a, anti-CD11c, and anti-CD18 MAbs. They were then washed and incubated with a detergent lysate of PBMC. After the washing, immobilized proteins were assayed for their ability to bind Biot-APG as described in Materials and Methods. Results depicted in Fig. 4 show that Biot-APG binds selectively to solubilized CD11b/CD18 and CD14 molecules retained in the wells with specific MAbs. Of note, Biot-APG bound only weakly to proteins immobilized on anti-CD11b (M1/70) and anti-CD14 (IOM2) MAbs, which compete for APG binding to the cell membrane. These observations indicate that APG bound to a site recognized by M1/70 on CD11b and to a site recognized by IOM2 on CD14. A very weak signal was observed in wells coated with BL5 (CD11a), and no signal was observed in wells coated with AFOL1 (CD11a) and IOMc (CD11c). A similar result was obtained with lysate from the human promyelocytic HL60 cell line treated for 48 h with phorbol myristate acetate (Fig. 4).

Involvement of CD14 in APG binding could also be demonstrated by using monocytes stripped of CD14 by pretreatment with PI-PLC, a phospholipase C which specifically cleaves phosphatidyl inositol linkage. Results in Table 2 indicate that nearly complete removal of CD14 from the monocyte surface decreased Biot-APG binding.

Internalization of APG in monocytes. A rapid decrease of membrane-associated APG fluorescence was observed in cells incubated at 37°C, down to 20% of initial values within 5 to 10 min, whereas membrane-associated Biot-APG remained stable in control cells (Fig. 5). Analysis by confocal microscopy revealed membrane and extracellular fluorescence in control cells kept at 4°C (Fig. 6a), intracellular patchy distribution after 5 min at 37°C (Fig. 6b), and intense irregular cytoplasmic staining after 60 min (Fig. 6c). These observations coupled with the flow-cytometric data indicate that APG was internalized by monocytes in a time-dependent manner.

Down-modulation of monocyte membrane antigens by APG. PBMC incubated for 45 min at 37°C in RPMI-FCS (2%) with or without (control samples) unlabelled APG at a saturating concentration were washed and labelled at 4°C with mouse MAbs. Results in Table 3 show that incubation with APG led to a significant down-modulation of the α-chain of the CR3 (37 to 39%) and CD14 (26 to 33%) molecules. APG also induced a slight decrease (15 to 19%) of CD18 expression, while

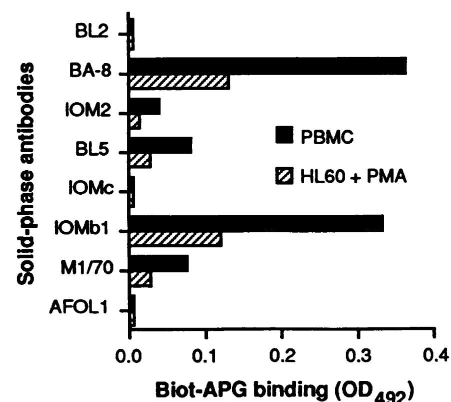

FIG. 4. Results of solid-phase binding assay with cell lysates. Wells of plastic plates were coated with the indicated MAbs as described in Materials and Methods, and detergent extracts of 5 × 10^7 PBMC or phorbol myristate acetate-treated HL60 cells (60 ng/ml, 48 h) were added and incubated for 1 h at 37°C. After a washing, wells received Biot-APG (5 μM) for 1 h and were washed again, and Biot-APG uptake was revealed by subsequent incubation with streptavidin-horseradish peroxidase and *o*-phenylenediamine. Results of one typical experiment were expressed as A_{492} after subtraction of background values (uncoated wells). OD_{492}, optical density at 492 nm.

TABLE 2. Decrease of specific binding of Biot-APG to monocytes after removal of CD14 molecules by PI-PLC[a]

PI-PLC (U/ml)	Biot-APG specific binding		LeuM3-FITC	
	ΔMFI[b]	% Decrease[c]	ΔMFI	% Decrease
0	124		70	
1.25	134	0	30	57
2.5	99	20	1	98

[a] PBMC were treated with PI-PLC at the indicated concentrations as described in Materials and Methods before staining with Biot-APG (10 μM) in the presence or absence of a 10-fold excess of unlabelled APG. Specific binding was calculated as described in Materials and Methods. LeuM3-FITC was used to control the removal of CD14 from monocytes.

[b] ΔMFI, MFI with background subtracted (MFI of cells incubated without labelled ligand).

[c] Percent decrease of labelled ligand binding calculated as 100 × (1 − ΔMFI of cells treated with PI-PLC/ΔMFI of cells treated with medium).

membrane CD11a, CD11c, HLA class I, and HLA class II antigens remained unchanged. Such results indicate possible cointernalization of CD11b antigen, whereas CD14 molecules were cointernalized and/or shed from the cell membrane.

Inhibition of APG binding by native and NaOH-hydrolyzed LPS. Knowing that CD11b and CD14 antigens mediate LPS binding to the cell membrane (43, 45), and on the basis of the structural similarities between APG and LPS (Fig. 1), we investigated the ability of LPS from rough (Re-LPS) and smooth (Kp-LPS and Ec-LPS) strains to compete with Biot-APG binding sites on human monocytes. Results in Table 4 show that, when solubilized in triethylamine buffer, smooth LPS from *K. pneumoniae* and rough LPS from *S. minnesota* slightly decreased Biot-APG binding.

Serum factors were previously reported to be required for specific binding of LPS to monocytes (9, 32). Binding in the presence of 5% FCS was therefore assessed, and the results (Table 4) show that LPS failed to compete for APG binding. Note that in the presence of serum, the ability of APG to compete with Biot-APG was significantly reduced (68 versus 91% inhibition with a 20-fold excess of unlabelled APG), indicating an increase of the nonspecific binding of Biot-APG. The polycationic polypeptide polymyxin B (PMxB) is known to associate with LPS by cation-anion interaction with the anionic lipid A and to neutralize the biological activities of LPS in vitro (26). Used at a high concentration, PMxB had a weak inhibitory effect (22 to 28%) on APG binding (Table 4). Finally, Kp-LPS and Ec-LPS were submitted to alkaline hydrolysis under the same conditions applied to *K. pneumoniae* membrane extracts to obtain APG (15, 17) and were used for competitive inhibition in a serum-free system. Results depicted as fluorescence histograms in Fig. 7 show that both NaOH-hydrolyzed LPS were strong competitors for Biot-APG binding to monocytes.

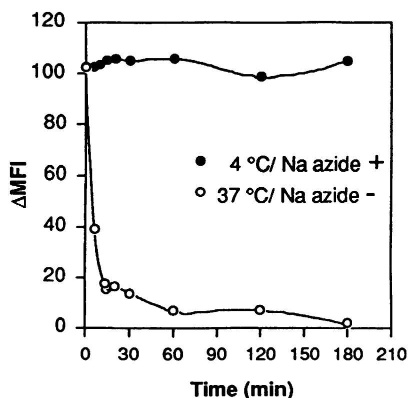

FIG. 5. Disappearance of APG from the cell surface at 37°C. Cells were labelled with 5 μM Biot-APG in RPMI 1640 for 30 min at 4°C, washed twice, and reincubated in RPMI 1640 with 2% FCS at 37°C or at 4°C in the presence of 0.1% Na azide (control cells). The decrease of membrane-bound APG at the indicated times was revealed with streptavidin-phycoerythrin.

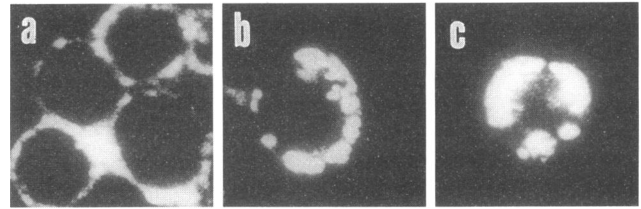

FIG. 6. Internalization of bound APG by human monocytes. Photographs represent the intracellular localization of APG visualized by confocal microscopy of an equatorial section of monocytes after staining with FITC-APG in RPMI 1640–2% FCS for 5 min at 4°C in the presence of sodium azide as a control (a), 5 min at 37°C (b), and 60 min at 37°C (c).

DISCUSSION

In this study, we report the binding of a purified APG isolated from *K. pneumoniae* membranes to human blood monocytes and we present direct evidence for the contribution of a ligand-receptor interaction. APG is a 34-kDa molecule obtained by alkaline hydrolysis, delipidation, and purification by preparative gel chromatography (15, 17). The overall structure of APG (Fig. 1) is that of LPS with galactose polymers as O antigen. Differences between APG and other LPS can be summarized as follows: (i) APG, though amphipathic, is highly hydrophilic whereas LPS is poorly soluble in water and tends to form complexes by lipid A-lipid A interaction, and (ii) APG bears only two amide-linked myristic acids (Fig. 1) whereas the lipid A moiety of LPS contains four to six fatty acids, some of them ester linked (28, 29).

The binding of APG was demonstrated by indirect immunofluorescence using Biot-APG revealed by streptavidin-phycoerythrin. With this method, we could show that Biot-APG binds to monocytes in a dose-dependent, saturable, and displaceable fashion in the absence of serum, while APG-specific binding was reduced in the presence of serum (Table 4).

The nature of the specific binding sites for APG on human monocytes was investigated in competitive binding experiments with MAbs to membrane molecules and in a solid-phase binding assay with proteins from monocyte lysates. Both methods indicate that specific APG binding was not mediated by the β-chain common to the CD11/CD18 family of adhesion molecules or by the α-chain of LFA-1 or p150,95 molecules because it was not inhibited by anti-CD11a, anti-CD11c, or anti-CD18 MAbs. However, the CD14 molecule and the α-chain of CR3 (CD11b) were shown to mediate APG-specific binding on the basis of competitive inhibition by appropriate MAbs and direct binding of Biot-APG to immobilized CD11b and CD14 molecules. Decreased APG binding to monocytes stripped of CD14 by PI-PLC confirmed the participation of CD14, a surface molecule linked to the lipidic membrane by a glycosylphosphatidyl-inositol bond.

The marked down-modulation of surface CD14 and CD11b (and, to a lesser extent, of CD18) during internalization of

TABLE 3. Down-modulation of monocyte membrane antigens by APG[a]

MAb/antigen	Expression of membrane antigen[b]						% Decrease[c]		
	Without APG			With APG (5 μM)					
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
IOT16/CD11a	78	73	77	78	78	79	0	0	0
IOMb1/CD11b	108	86	102	63	54	64	39	37	38
IOMc/CD11c	69	84	68	68	69	68	2	2	1
60.3/CD18	91	79	90	76	64	76	16	19	15
IOM2/CD14	62	79	61	41	59	42	33	26	32
W6.32/HLA I	71	76	70	72	71	72	0	7	0
BL2/HLA II	85	78	85	84	63	85	1	2	0
3G8/RFCγIII	67	ND[d]	66	71	ND	71	0	ND	0

[a] PBMC (10^6 in 1 ml of RPMI 1640 containing 2% FCS) were incubated for 45 min at 37°C in the presence or absence of unlabelled APG and then washed twice and stained with mouse MAbs against membrane antigen. Membrane-bound MAbs were revealed by subsequent incubation with FITC-F(ab')$_2$ of goat anti-mouse immunoglobulin.
[b] Results are expressed as ΔMFI after subtraction of MFI values for samples stained with the irrelevant antibody 8545A.
[c] Percent decrease of membrane antigen expression calculated as 100 × (1 − ΔMFI in the presence of APG/ΔMFI in the absence of APG).
[d] ND, not determined.

APG at 37°C is further indirect evidence for their association with APG on the cell membrane (Table 3).

The first membrane molecule previously shown to interact with LPS is the CD11b/CD18 integrin. Wright and coworkers demonstrated the formation of rosettes between human monocytes and sheep erythrocytes coated with LPS or lipid IVA and their inhibition by MAbs to CD11b/CD18 (42, 43). Rosettes could be formed in the absence of serum but not in the absence of Ca^{2+} and Mg^{2+} at 37°C (43). Similarly, the binding of *Bordetella pertussis* LPS to mouse macrophages was reported to be strongly reduced in the presence of Ca^{2+} and Mg^{2+} chelating agents (32). Calcium and magnesium stabilize surface integrins and contribute to several of their functions (28, 31). Since our experiments were performed at 4°C, we cannot exclude a possible stabilizing effect of divalent cations at 37°C. Two groups of investigators excluded a contribution of CD11b/CD18 to LPS binding (4, 5). In fact, this conclusion was drawn from the inability of five anti-CD11b MAbs (60.1, MN41, 4B3, Leu15, and 44A) to inhibit LPS binding in the presence of serum (5) or in the absence of serum in the case of MAbs Leu15 and 44A (4). In view of the exquisite epitope specificity demonstrated here with APG, it is possible that those antibodies did not react with the N-terminal lectin-like region recognized by anti-Mac1 (M1/70) and anti-Mo1 MAbs (36). However, the integrin CD11b/CD18 is not the only functional LPS receptor because cells from patients with leukocyte adhesion deficiency still respond to LPS in vitro (41).

TABLE 4. Mild inhibition of Biot-APG binding to monocytes by native LPS[a]

Competitor and concn	Biot-APG binding			
	Without FCS		With FCS (5%)	
	ΔMFI[b]	% Decrease[c]	ΔMFI	% Decrease
None	76		70	
APG				
100 μM	20	74	35	50
200 μM	7	91	23	68
Kp-LPS				
1 mg/ml	76	0	74	0
2 mg/ml	61	19	76	0
Re-LPS				
1 mg/ml	73	4	73	0
2 mg/ml	61	19	66	6
PMxB				
200 μM	59	22	ND[d]	ND
400 μM	55	28	ND	ND

[a] Results for one experiment selected from three showing similar results. PBMC were incubated for 30 min at 4°C in 40 μl of binding buffer with or without FCS containing Biot-APG (10 μM final concentration) in the absence (control sample) or presence of APG, native LPS solubilized in triethylamine (0.5%), or PMxB. Cells were washed twice and bound Biot-APG was revealed as described in Materials and Methods.
[b] Calculated as described in Materials and Methods.
[c] Percent decrease of Biot-APG binding calculated as 100 × (1 − ΔMFI in the presence of competitor/ΔMFI in the absence of competitor).
[d] ND, not determined.

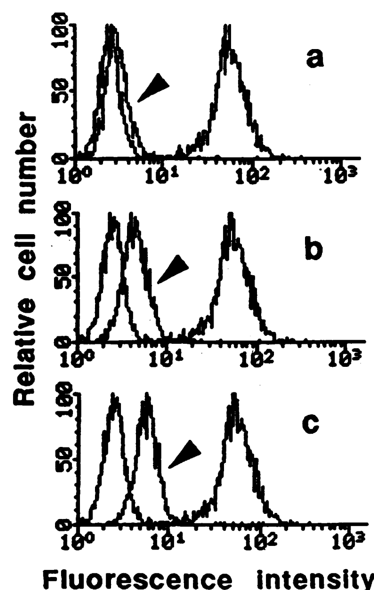

FIG. 7. Competitive inhibition of Biot-APG by LPS(NaOH). PBMC were incubated with Biot-APG (5 μM) in the presence of a 10-fold excess of unlabelled APG or 2 mg of LPS(NaOH), and binding of Biot-APG was determined as described in Materials and Methods. Results were expressed as fluorescence histograms (right histogram, fluorescence of cells incubated with Biot-APG in the absence of competitor; left histogram, background fluorescence). Arrows indicate the fluorescence shifts of cells stained in the presence of APG (a), Kp-LPS(NaOH) (b), and Ec-LPS(NaOH) (c).

A second cell surface structure reported to interact with LPS is the 55-kDa glycosylphosphoinositol-anchored CD14 molecule. Recent studies reported the fixation of LPS to CD14 on a macrophage cell line (20) and on neutrophils (25) and to recombinant soluble CD14 (11) in the absence of serum, as is the case for APG. However, the binding to CD14 is markedly enhanced when LPS is complexed with LPS-binding protein (LBP) (11, 20, 25, 45) or with septin (44), two proteins found in normal serum. In view of the structural similarity between LPS and APG, the present data strongly suggest that CD14 may directly interact with APG. Such binding could be amplified by LBP in the case of native LPS, whereas there is no evidence for the contribution of a serum protein in APG binding to CD14. Couturier et al. reported that the binding of *Neisseria meningitidis* LPS to CD14 was enhanced in the presence of heat-inactivated FCS (5) which does not contain functional LBP, suggesting that other factors may control LPS-CD14 interactions. Since IOM2 MAb blocks the binding of *N. meningitidis* LPS (5) and that of APG to monocytes, we suggest that APG and LPS bind without intermediate protein to one epitope recognized by IOM2 MAb on the CD14 molecule. In addition, monocyte cytokine synthesis induced by LPS in serum-free medium and human immunodeficiency virus type 1 expression from an LPS-stimulated monocytic cell line (granulocyte macrophage-colony-stimulating factor-treated U937 line) in the absence of LBP were blocked by anti-CD14 MAbs (1, 6). Recently, Kitchens et al. (20) demonstrated that the MAb 60b, which blocks the activities of the LPS-LBP complex, also blocks the direct binding of LPS and the subsequent NF-κB response of the human monocyte/macrophage THP-1 cell line. Altogether, the data support the hypothesis of direct binding of LPS to CD14 rather than an interaction between CD14 and LBP (11). LBP could alter the conformation of the LPS molecule in a way that facilitates LPS-CD14 binding.

By using several *K. pneumoniae* derivatives which differ from each other at precise regions of the LPS-like structure, we were able to obtain indirect information regarding the sites of APG which interact with the monocyte surface. The poly(1-3)D-galactose chain (GC-APG) does not inhibit APG binding (Fig. 3), indicating a lack of interaction between the O antigen and the monocyte surface, in agreement with other studies (20, 26, 32). Additional ester-linked palmitates and myristates in the APG molecule (EFA-APG) do not modify APG binding. These observations are in agreement with our previous study (17) in which biotinylated antibodies specific for the polygalactose chain were used to demonstrate the absence of GC-APG binding to monocytes and the identical binding capacities of APG and EFA-APG. The PP_i at position C-1 was not essential in the interaction of APG with the monocyte membrane because MP-APG preserved all the capacity of unlabelled APG to inhibit Biot-APG binding (Fig. 3). By exclusion, it appears that the diglucosamine phosphorylated at position C-4′ is the minimal structure required for the association with membrane receptors. CD11/CD18 molecules appear to recognize glucosamine phosphate of the lipid IVA molecule exposed on the sheep erythrocyte surface (42) as well as the soluble phosphosugars extracted from *Histoplasma capsulatum* (3) and from *Leishmania mexicana* (34). In regard to the two βOH-myristates of APG, they may contribute through hydrophobic interactions with membrane lipids to stabilization of the binding initiated by specific ligand-receptor interactions. However, Tahri-Jouti et al. suggested that fatty acid esters may be directly involved in the binding of *B. pertussis* LPS to mouse macrophages (33).

The possible implication of the LPS core region in the binding to surface receptors could not be analyzed with the set of APG derivatives used in this study. Haeffner-Cavaillon et al. (10) suggested a lectin-like receptor for the core domain of LPS, and more recently Lei and Morrison (23) have identified in the macrophagic cell line J774 a 38-kDa molecule specific for the inner core region (KDO) determinant of the LPS. Our competitive inhibition experiments with GC-APG do not exclude the participation of the core region in binding because GC-APG contains only 30% of the total KDO of APG (15, 17), while interaction of the core region with cell membranes requires an intact KDO at the reducing end of the polysaccharide moiety (8, 10).

Despite their comparable overall structures, LPS and APG do not seem to have identical properties of binding to the monocyte membrane, as shown by the poor ability of smooth (*K. pneumoniae* and *E. coli*) and rough (*S. minnesota* Re) LPS to compete for Biot-APG binding to monocytes in the absence of serum, while no competition could be demonstrated in the presence of serum (Table 4). However, alkaline hydrolysis converts LPS into a strong competitor for APG binding (Fig. 7). Removal of the ester-linked fatty acids by alkaline hydrolysis does not account for these changes because EFA-APG displays binding properties identical to those of APG. It is certain that the hydrophobic parts of APG and LPS (26, 40) are the prevalent substructures involved in the binding to cell membranes. Therefore, the distinct binding properties of APG and LPS can be related to discrete structural differences between their respective hydrophobic regions. We hypothesize that alkaline treatment of *K. pneumoniae* membrane extract converts the LPS molecule into a highly hydrosoluble Na salt form and decreases the anionic charge. However, the cationic antibiotic PMxB still partially decreased APG binding to monocytes (Table 4) but failed to inhibit the monocyte oxidative burst induced by EFA-APG. In conclusion, alkaline hydrolysis and partial deacylation markedly enhance the binding properties while abrogating the capacity to induce cellular responses (15, 16).

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