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Monocyte cytokine secretion induced by chemically-defined derivatives of *Klebsiella pneumoniae*

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SUMMARY

The capacity of a *K. pneumoniae* membrane proteoglycan (Kp-MPG) and four of its chemically defined derivatives to activate human monocytes was studied by measuring immunoreactive IL-1β, IL-6 and tumour necrosis factor-alpha (TNF-α) in culture supernatants. Monocyte culture supernatants were also tested for their comitogenic activity on concanavalin A-stimulated thymocytes and for their cytotoxic activity on the mouse fibroblastic L929 cell line. The four Kp-MPG derivatives were: (i) an acylpoly(1–3)galactoside (APG); (ii) an APG preparation submitted to acid hydrolysis which removed all fatty acids but left intact the galactose chain of APG (GC-APG); (iii) a preparation obtained by mild alkaline hydrolysis, containing additional ester-linked C_{14} and C_{16} fatty acids bound to the APG molecule (EFA-APG); and (iv) a polymer of the latter compound (APG pol). Kp-MPG induced the synthesis of IL-1β, IL-6 and TNF-α with dose–responses and kinetics similar to those of *Salmonella minnesota* lipopolysaccharide (Sm-Re-LPS). APG pol and EFA-APG induced the secretion of the three cytokines with lower potency than Kp-MPG or Sm-Re-LPS. APG did not trigger any detectable cytokine production and GC-APG induced only borderline and inconsistent responses. Our data demonstrate the critical role of ester-linked C_{14} and C_{16} fatty acids in the triggering of monocyte response to Kp-MPG derivatives.

Keywords IL-1β IL-6 tumour necrosis factor-alpha *Klebsiella pneumoniae* ester-linked fatty acids

INTRODUCTION

Attachment of Gram-negative bacteria to mononuclear phagocytes induces phagocytosis and triggers the synthesis of various cytokines including tumour necrosis factor-alpha (TNF-α), IL-1 and IL-6 [1]. Lipopolysaccharide (LPS), the major component of the outer wall of Gram-negative bacteria, has been extensively investigated and the contribution of defined LPS submolecular structures to the binding to the cell surface receptors and the triggering of cellular responses is progressively emerging [2,3]. A better understanding of the structure–activity relationship within the LPS molecule will permit the development of selective antagonists as well as immunomodulators which would retain the capacity to stimulate non-specific immunity without toxic side effects. Although there are accumulated data on LPS, little is yet known about other components of bacterial membrane. A proteoglycan from a non-encapsulated strain of *K. pneumoniae* (Kp-MPG) was reported to stimulate natural killer (NK) cell activity [4], IL-1 [5] and IL-6 synthesis [6] as well as to be a polyclonal B cell activator [5,7]. Kp-MPG in combination with ribosomal preparations of *K. pneumoniae*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *S. pyogenes* has been in clinical use for many years for treatment of respiratory tract infections [8]. Subsequently, several derivatives of Kp-MPG were prepared and characterized. One of them, an acyl-poly(1-3)galactoside (APG) is presently evaluated for its capacity to label inflammatory and tumour foci when administered as a ^{99}Tc conjugate [9]. Here we report the relative capacity of these derivatives to trigger IL-1β, IL-6 and TNF-α secretion by human monocytes. The availability of several preparations of different chemically defined compounds enabled us to address the role of each structure in the triggering of the cellular responses.

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MATERIALS AND METHODS

Stimuli

LPS from *Salmonella minnesota* Re (Sm-Re-LPS) was obtained from Sigma (St Louis, MO). A membrane proteoglycan from a non-encapsulated strain of *K. pneumoniae* (Institut Pasteur I-145) (Kp-MPG) was prepared as already described [4]. Four different derivatives were prepared from Kp-MPG. Their

Table 1. Analytical composition of the four Kp-MPG derivatives

	EFA-APG	APG pol	APG	GC-APG
Galactose	65	62	70	87
Ketodeoxyoctonic acids	3·0	3·0	3·0	1·0
Glucosamines	5·4	4·3	3·9	2·0
Aminoacids	9·1	10·2	0·97	0·5
C14βOH-myristic acids (amide-linked)	2·09	2·8	1·62	0
Ester-linked C_{14} fatty acids	0·68	0·7	0	0
Ester-linked C_{16} fatty acids	0·85	0·9	0	0

Content expressed as percentage of total mass (not corrected for water content).

analytical composition is presented in Table 1. A first preparation was made by mild alkaline hydrolysis (NaOH 0·1 *N*, 24 h, 22°C) followed by preparative gel chromatography yielding two fractions: a homogeneous 34-kD fraction containing ester-linked fatty acids (EFA-APG) and a 100-kD component (APG pol) which was characterized as a polymer of EFA-APG. A second preparation was obtained from Kp-MPG by two cycles of drastic alkaline hydrolysis (NaOH 0·5 *N*, 1 h, 56°C) with a delipidation (chloroform/methanol 3:1 v/v) followed by gel chromatography on Sephacryl S200 HR with Tris–HCl–EDTA buffer 0·01 M, pH 7·4, yielding the 34-kD fraction APG. This fraction was submitted to acid hydrolysis and the non-precipitable material which contained mainly poly (1-3) galactose chains but no fatty acids was designated as GC-APG.

The detailed chemical structure of Kp-MPG will be presented in a separate report (manuscript submitted). The common structure of all 34-kD derivatives is an homopolysaccharidic chain consisting of 150 1-3-linked galactose residues, terminated by a core-like zone and a lipid part containing two glucosamine residues bound to phosphate groups and to $C_{14}\beta$OH myristic acids by amide links.

EFA-APG differs from APG by the presence of additional ester-linked C_{14} myristic acids and a C_{16} palmitic acid. These ester-linked fatty acids are present in higher amounts in APG pol. GC-APG lacks the biphosphodiglucosamine residues, and all the fatty acids.

Preparation of monocytes and cell cultures

Blood samples of healthy volunteers (Centre de Transfusion, Lyon, France), collected in heparin (Roche, Neuilly-sur-Seine, France) (20 U/ml), were fractionated by centrifugation on a cushion of Ficoll–Hypaque (Lymphoprep, Flobio, Paris, France). Mononuclear cells were harvested from the interface Ficoll–plasma, diluted in HBSS (Vietech, St Bonnet de Mure, France) and centrifuged at 4°C for 10 min (400 ***g***) followed by two washes in HBSS for 10 min at 100 ***g*** to remove the platelets. Cells were resuspended in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% selected heat-inactivated fetal calf serum (Seromed, Noisy-le-grand, France), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Monocyte percentage in the total monunuclear cells was determined after staining with fluoresceinated anti-CD14 antibody (anti-LeuM3; Becton Dickinson, Mountain View, CA) and cytofluorometric analysis. Cell suspensions (5×10^6/ml) were cultured in 1-ml wells of culture plates (Costar, Cambridge, MA) for 90 min at 37°C in humidified air with 5% CO_2. Non-adherent cells were discarded and medium with or without appropriate stimulus was added. After 24 h, unless otherwise specified, cell-free supernatants were harvested, centrifuged (10 000 ***g***) and stored at −20°C until use. All media and serum were selected for endotoxin contamination below the threshold of the *Limulus* amoebocyte lysate test.

Cytokine immunoassays

Cytokine concentration in cell culture supernatants was assessed by an ELISA sandwich-type method with solid-phase MoAb and revelation by a polyclonal antibody followed by a peroxidase conjugated anti-immunoglobulin antibody. The detailed procedure was adapted from a previously described ELISA [10]. The recombinant cytokines rTNF-α (ref. BBG4), rIL-1β (ref. BIL-1-C) and rIL-6 (ref. 1542-00) were purchased from Genzyme (Boston, MA) and used for calibration. Murine MoAbs specific for hTNF-α (clone BC7), hIL-1β (clone BA15) and hIL-6 (clone BE4) were from the Centre de Transfusion Sanguine, Besançon, France. Polyclonal rabbit anti-hTNF-α (ref. BO405) and anti-hIL-1β (ref. IP300) were purchased from Genzyme. They were revealed by peroxidase-labelled goat anti-rabbit Fc (Jackson, Stanford, CA). Goat anti-hIL-6 antibodies were obtained from Janssen (Beerse, Belgium) and revealed by donkey anti-goat immunoglobulin antibody (Jackson). The specificities of the MoAbs for the three mentioned cytokines were controlled by testing their cross-reactivity with the two other cytokines and with recombinant interferon-gamma (rINF-γ) and rIL-2 (both from Genzyme; ref. HG-INF and RIL-2, respectively).

Measurement of IL-1 and TNF-α activities

IL-1 activity in the supernatants of stimulated adherent monocytes was evaluated by the original murine thymocyte proliferation assay of Gery *et al.* [11]. Thymocytes from 6-week-old C3H/HeJ mice (IFFA Credo, l'Arbresle, France) were stimulated by concanavalin A (1 μg/ml) and various dilutions of monocyte supernatants, then ^{3}H-thymidine incorporation was determined after 60–72 h of culture [12]. For measurement of TNF activity the mouse fibroblastic cell line L929 (American Type Culture Collection, Rockville MD) was exposed to monocyte culture supernatants at doubling dilutions for 18 h in presence of actinomycin D 1 μg/ml and the uptake of crystal violet by viable adherent cells was monitored by photometry at 540 nm [13].

RESULTS

The detection limit and reproducibility of each ELISA was ascertained; the ranges for IL-1β, IL-6 and TNF-α were 0·6–20 ng/ml; 0·25–15 ng/ml; and 0·10–4 ng/ml, respectively. Inter-assay precision (six experiments) was below 10% for IL-6 and TNF-α assays, and below 15% for IL-1β assay. The dilution curves of cell culture supernatants of activated adherent monocytes were parallel to standard curves established with recombinant cytokines (Fig. 1). The specificity of each assay was checked by addition of rIL-1β, rIL-6, rTNF-α, rIL-2 and rINF-γ at the highest concentrations encountered in supernatants of activated monocytes or lymphocytes. A slight interference was detected only with rIL-2 in the IL-1 assay; the absorbance

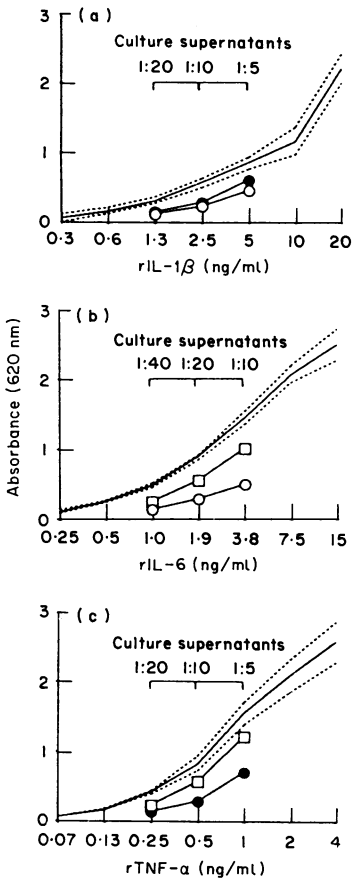

Fig. 1. ELISA for IL-1β (a); IL-6 (b); and TNF-α (c). Mean values and inter-assay coefficient of variation in absorbance at 620 nm obtained with recombinant cytokines (standard curves) in six experiments. Example of titration curves of supernatants from adherent monocytes stimulated by Kp-MPG (□), APG pol (●) or EFA-APG (○).

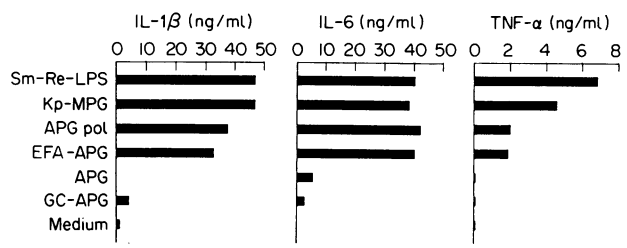

Fig. 2. Secretion of IL-1β, IL-6 and TNF-α induced by Sm-Re-LPS, Kp-MPG (10 μg/ml) and its derivatives (100 μg/ml). Cytokine levels were measured in 24-h supernatants by ELISA.

measured with 20 ng/ml of rIL-2 was equivalent to that of 0·01 ng of IL-1β.

In a first experiment we determined the capacity of each stimulus introduced at a single concentration to trigger IL-1β, IL-6 and TNF-α secretion. Results presented in Fig. 2 show that Sm-Re-LPS and Kp-MPG are potent stimuli. APG pol and EFA-APG induced a strong response whereas APG and GC-APG failed to stimulate IL-1β, IL-6 and TNF-α secretion.

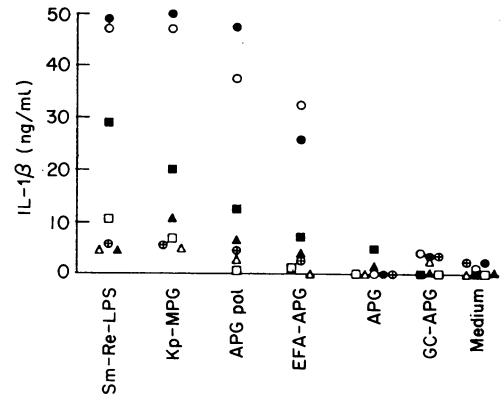

Fig. 3. Distribution of IL-1β concentrations in supernatants of adherent monocytes from seven different healthy individuals. Stimuli were added at 10 μg/ml for Sm-Re-LPS and Kp-MPG and 100 μg/ml for Kp-MPG derivatives.

Knowing that cytokine secretion by human monocytes may vary considerably among different individuals, we repeated the experiment with seven blood donors. Before adherence the percentage of LeuM3$^+$ cells (CD14$^+$) ranged between 17 and 21%. Such cells were not detectable in the non-adherent cell population. Levels of IL-1β secretion are presented in Fig. 3. As expected, responses to Sm-Re-LPS and Kp-MPG were quite variable, ranging from 4·4 to 50 ng/ml. Monocytes from five out of seven donors were stimulated by APG pol and EFA-APG but none of them responded to APG. A very weak response was observed with GC-APG, never exceeding twice the background level. Measurements of IL-6 and TNF-α in these supernatants revealed the same order of potency of the different stimuli (data not shown). Dose–responses were studied with Kp-MPG, APG pol and EFA-APG (Fig. 4). Maximum cytokine secretion was induced at 10–100 μg/ml but a strong response was already detected at 1 μg/ml. These data justified and confirmed the use of Kp-MPG derivatives at 100 μg/ml in previous experiments.

Kinetics of cytokine secretion were analysed using supernatants harvested 3–24 h after addition of stimulus (Fig. 5). Sm-Re-LPS and Kp-MPG induced a rapid secretion of IL-1β, already detectable at 3 h and reaching a plateau after 12 h. The response to APG pol and EFA-APG was slightly delayed. Secretion of IL-6 was significant at 3 h and continued up to 24 h. Secretion of TNF-α occurred rapidly with a high level at 3 h and plateaued after 6–12 h. Finally, in order to ascertain that cytokines assayed in supernatants resulted from the *de novo* synthesis by adherent monocytes, we performed cultures in presence of cycloheximide, a protein synthesis inhibitor. At 10 and 100 μg/ml, cycloheximide completely blocked IL-6 secretion (Fig. 6).

Biological activity of cytokines in the supernatants of activated monocytes was demonstrated by potentiation of C3H/HeJ thymocyte proliferation and by the cytotoxic effect on the mouse fibroblastic L929 cell line, indicating IL-1-like and TNF activities (Fig. 7). Results obtained in the two bioassay systems were comparable to the effects of purified recombinant molecules.

DISCUSSION

We have investigated the capacity of Kp-MPG and four of its derivatives to activate human adherent monocytes. The avail-

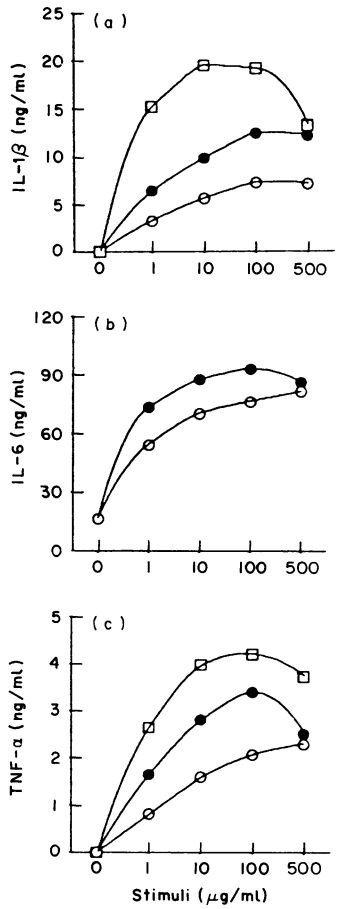

Fig. 4. Dose-response of IL-1β (a); IL-6 (b); and TNF-α (c) to stimulation by Kp-MPG (□), APG pol (●) and EFA-APG (○).

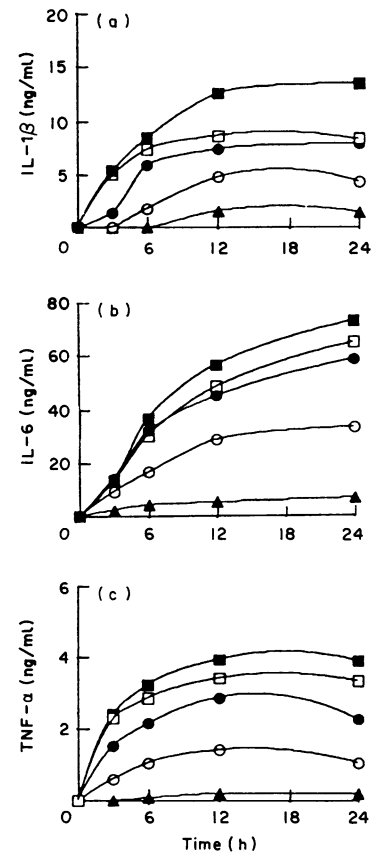

Fig. 5. Kinetics of IL-1β (a); IL-6 (b); and TNF-α (c) secretion by adherent monocytes stimulated with Sm-Re-LPS (10 μg/ml) (■), Kp-MPG (10 μg/ml) (□), APG pol (100 μg/ml) (●), EFA-APG (100 μg/ml) (○) or medium (▲).

ability of several compounds bearing defined chemical modifications enabled us to study the contribution of different submolecular structures of these derivatives to the triggering of monocyte activation. We show that Kp-MPG induced a rapid dose-dependent secretion of IL-1β, IL-6 and TNF-α. This observation is in agreement with previous reports that demonstrated that the same compound triggered IL-6 production by human monocytes [6] and IL-1 secretion by murine spleen cells [5]. The specificity and reproducibility of the ELISAs were satisfactory for their application to measurement of cytokine concentrations in the supernatants of activated adherent monocytes. The possible interference of IL-1α in the IL-1β assay has not been extensively studied, but IL-1α is unlikely to be secreted in large amounts by activated monocytes. Demonstration of immunoreactive IL-1, IL-6 or TNF-α does not necessarily indicate that these cytokines are biologically active because IL-1 receptor antagonist, soluble receptors and other ligands may also be secreted by activated monocytes. However, we could demonstrate co-mitogenic activity on concanavalin A-stimulated C3H/HeJ thymocytes, indicating the presence of biologically active IL-1. Furthermore, titration curves of TNF activity, using the L929 fibroblast cell line, demonstrated a strict parallelism between dilutions of rTNF-α and supernatants, thus excluding the presence of blocking factors in monocyte supernatants.

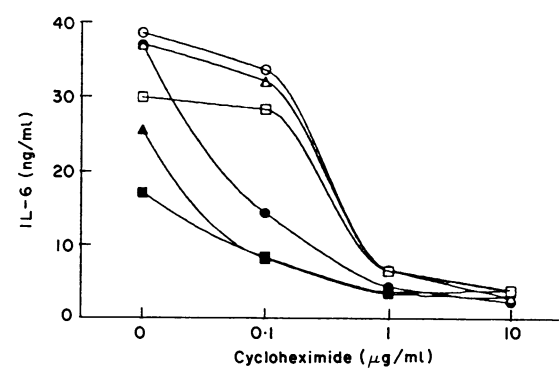

Fig. 6. IL-6 secretion by adherent monocytes stimulated by Kp-MPG (circles), APG pol (triangles) or EFA-APG (squares) in the presence of cycloheximide. Solid symbols, 3-h culture; blank symbols, 6-h culture.

The galactose chain which represents the predominant part of the 34-kD Kp-MPG derivatives did not contribute significantly to monocyte activation, as shown by the very low or absent induction of IL-1β, IL-6 and TNF-α secretion by GC-APG which bears an intact poly(1-3)galactose chain but lacks the lipid part and most of the core region of APG. This result

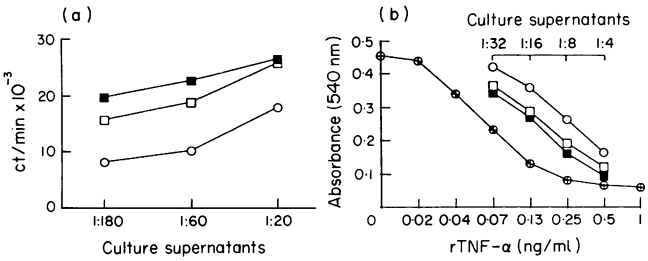

Fig. 7. IL-1 (a) and TNF (b) activities in culture supernatants of adherent monocytes stimulated for 20 h with 10 μg/ml Kp-MPG (□) and 100 μg/ml of APG Pol (●) or EFA-APG (○). Supernatants were tested at the indicated dilutions for their co-mitogenic activity on C3H/HeJ thymocytes (a) and their cytotoxic activity on L929 fibroblasts (b). (a) ^{3}H-thymidine uptake as ct/min × 10^3; mean values of triplicate cultures. Con A-stimulated controls without exogenous IL-1 incorporated 2828 ± 490 ct/min; (b) absorbance at 540 nm of crystal violet incorporated into viable adherent fibroblasts.

was expected, since this molecule did not bind to monocytes, although it might interact with the Mac-2 antigen, a macrophage lectin specific for galactose which is expressed on activated macrophages [14] but very weakly or not at all on monocytes [15]. Conversely, other polysaccharides, as for instance the PS-1 from *Bordetella pertussis*, were shown to interact with monocyte membrane structures and to activate IL-1 secretion [16].

The most striking observation was the lack of monocyte activation by APG in contrast to the induction of IL-1β, IL-6 and TNF-α secretion by EFA-APG and its polymer APG pol. The main difference between these compounds lies in the drastic alkaline hydrolysis and the delipidation steps used for the preparation of APG, which remove the ester-linked C_{14} and C_{16} fatty acids present in EFA-APG and APG pol. Therefore, these ester-linked fatty acids appear to be critical in the triggering of monocyte activation. It is noteworthy that the ester-linked fatty acids do not contribute to the attachment of Kp-MPG derivatives to monocytes membrane since we have found that APG and EFA-APG displayed identical binding capacities to human monocytes [17]. Thus distinct molecular substructures are involved in the binding to membrane receptors and the triggering of cellular responses.

Our data are in agreement with the structure–activity studies of LPS molecules. The lipidic part of LPS seems to be critical for induction of cellular responses as suggested by the loss of macrophage activation after incorporation of LPS into liposomes [18,19], although one group of investigators reported data suggesting a role for the core region [20,21], while others could not demonstrate any activity of synthetic 3-deoxy-D-manno-2-octulosonic acid (KDO) compounds devoid of fatty acids [22]. Our results with Kp-MPG derivatives emphasize the role of the ester-linked fatty acids, in complete agreement with the well-documented correlation between the degree of acylation of synthetic lipid A analogues and their biological activity [23,24].

We show that induction of monocyte activation by Kp-MPG derivatives is associated with the presence of ester-linked C_{14} and C_{16} fatty acids. The APG molecule, which lacks these ester-linked fatty acids, was shown to be suitable for *in vivo* labelling of macrophages in inflammatory foci [9], suggesting that this compound retained its capacity to bind to cells of the monocytes/macrophage lineage without triggering the secretion of inflammatory cytokines.

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