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Similar Mechanisms of Action of Defined Polysaccharides and Lipopolysaccharides: Characterization of Binding and Tumor Necrosis Factor Alpha Induction

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Little has been reported about the effects of different polysaccharides on cytokine production from human monocytes. In this study, we show that several well-defined polysaccharides, including polymers with different sizes of β1-4-linked D-mannuronic acid (poly-M, high-M alginate, and M-blocks) and cellulose oxidized in the C-6 position, induced human monocytes to produce tumor necrosis factor alpha (TNF-α). Poly-M was the most efficient polysaccharide tested and, on a weight basis, was approximately as efficient as lipopolysaccharide (LPS) from *Escherichia coli*. TNF-α production was shown to depend strongly on the molecular weights of poly-M and high-M alginate, with maximal TNF-α production occurring at molecular weights above 50,000 and 200,000, respectively. G-blocks, α1-4-linked L-guluronic acid polymers that did not induce cytokine production from monocytes, reduced the cytokine production induced by the β1-4-linked polyuronic acids and LPS. Furthermore, both G-blocks and LPS were found to inhibit the binding of poly-M to monocytes, as measured by flow cytometry. In addition, we found that the binding of LPS to monocytes was inhibited by G-blocks, M-blocks, and poly-M. Our results indicate that β1-4-linked polyuronic acids and LPS may stimulate monocytes to produce TNF-α by similar mechanisms and may bind to a common receptor.

Monocytes can be stimulated by various agents, including bacterial lipopolysaccharide (LPS), to produce the cytokines tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and IL-6 (1, 3, 28). LPS contains polysaccharide and lipid A portions, and much of the toxic and immunomodulating activities of LPS are reported to be mediated by the lipid A portion (13, 26). On the other hand, the polysaccharide portion has been implicated in both the binding of LPS to cells and cytokine production from monocytes (7, 27, 34). We previously showed that short polymers (blocks) of β1-4-linked D-mannuronic acid (D-ManA) derived from algae stimulated monocytes to produce TNF-α, IL-1, and IL-6, whereas blocks of α1-4-linked L-guluronic acid (L-GulA) did not induce cytokine production (32). Furthermore, polysaccharides such as β1-3-glucans (β1-3-linked D-glucose [D-Glc]) have been reported to have antitumor activity and to stimulate monocytes to produce cytokines (36). The immunostimulating activities of polysaccharides could be of importance for the treatment of neoplastic as well as infectious diseases (8, 10). Little is known about the structural requirements of polysaccharides for optimal cytokine stimulation.

The present investigation was undertaken to study the relationship between polyuronic acids and LPS with regard to TNF-α-inducing abilities and binding to monocytes. We found that the β1-4-linked polyuronic acids tested stimulated monocytes to produce TNF-α and that longer polymers of β1-4-linked D-ManA (poly-M) produced by *Pseudomonas aeruginosa* and LPS from *Escherichia coli* were approximately equally efficient. Blocks of α1-4-linked L-GulA inhibited the cytokine-stimulating activities of the other polyuronic acids and LPS. These data demonstrate that β1-4-linked polyuronic acids are very potent cytokine inducers in vitro and that there are similarities between LPS and β1-4-linked polyuronic acids with regard to the stimulation of TNF-α production from monocytes. Furthermore, the binding of poly-M and LPS to monocytes, as measured by flow cytometry, was inhibited by both G-blocks and LPS, suggesting a common binding molecule(s) for the different polyuronic acids and LPS on the monocyte surface.

MATERIALS AND METHODS

Polysaccharides. The monomer composition, sequential arrangement, and degree of polymerization of G-blocks, M-blocks, and high-M alginate were analyzed by ^{1}H nuclear magnetic resonance spectroscopy on a Bruker 400 WM spectrometer as described previously (15, 16).

M-blocks (95% D-ManA and degree of polymerization, 30 to 35) and high-M alginate (85% D-ManA) were isolated from the intracellular substance of *Ascophyllum nodosum* fruiting bodies as described by Haug et al. (20). From long polymers of high-M alginate (molecular weight [MW], 600,000), samples with different MWs were prepared by partial hydrolysis for 10 to 120 min at 100°C and pH 5.4. The average MWs were estimated at 20°C from intrinsic viscosity measurements of polysaccharide solutions in sodium chloride (0.1 M) by use of the Mark-Howink-Sakurada relation (19) and a Ubbelohde suspended-capillary viscometer with an automatic dilution viscosity system (Scott-Geräte).

G-blocks (>90% L-GulA and degree of polymerization, 25 to 30) were isolated from the alga *Laminaria digitata* (20).

High-MW homopolymeric β1-4-linked D-ManA (poly-M) was isolated from agar colonies of *P. aeruginosa* DE 127 grown at low temperatures as described previously (38). A

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TABLE 1. Polysaccharides used in these experiments

Polysaccharide	Major linkage(s)	MW	Monomer composition
Poly-M	β-1-4	70,000[a]	>99% D-ManA
High-M alginate	β-1-4	6,000–550,000	85% D-ManA, 15% L-GulA
M-blocks	β-1-4	6,000–7,000	95% D-ManA, 5% L-GulA
C6OXY	β-1-4	60,000	94% D-GlcA, 6% D-Glc
G-blocks	α-1-4	5,000–6,000	90% L-GulA, 10% D-ManA
Hyaluronic acid	β-1-4 and β-1-3	1,200,000	50% D-GlcA, 50% D-GlcNAc

[a] Samples used for experiments shown in Fig. 2 and Tables 2 and 5.

400-MHz ^{1}H nuclear magnetic resonance spectrum of poly-M showed that more than 99% of the polysaccharide content was D-ManA (38). From this high-MW poly-M, samples with different chain lengths were prepared by partial hydrolysis for 10 to 120 min at 100°C and pH 5.4. The average MWs were estimated from intrinsic viscosity measurements (19).

C6OXY (β1-4-linked glucuronic acid [D-GlcA]) was prepared by oxidation of cellulose at position C-6 (13a). The average MW was estimated from intrinsic viscosity measurements to be 60,000, and the degree of oxidation (94% D-GlcA and 6% D-Glc) was determined by titration (31, 43).

Hyaluronic acid, consisting of repeating D-glucuronic acid-β1-3-*N*-acetylglucosamine units linked by β1-4 bonds and with an average MW of 1,200,000, was obtained from Food Chemifa, Kibun Springs, Japan.

Table 1 and Fig. 1 provide an overview of the polysaccharides used in these experiments.

E. coli-derived LPS (Sigma) (O26:B6) was used in these experiments unless otherwise noted. LPS from *P. aeruginosa* (Sigma) (serotype 10) was also used.

Endotoxin contamination in the different polysaccharides was measured by the *Limulus* amebocyte lysate (LAL) assay (Kabi Vitrum, Stockholm, Sweden). The estimated levels of endotoxin were as follows: M-blocks, 24 pg/μg; high-M alginate, 43 pg/μg; poly-M, 14 ± 6 ng/μg; G-blocks, 23 pg/μg; C6OXY, 70 pg/μg; and hyaluronic acid, 21 pg/μg.

Monocyte cultivation. Mononuclear cells were isolated from human A$^+$ blood buffy coat (The Bloodbank, University of Trondheim, Trondheim, Norway) as described by Bøyum (5). The adherent cell population of the mononuclear cells was cultured in 16-mm-well culture plates (Costar, Cambridge, Mass.) containing RPMI 1640 (GIBCO, Paisley, United Kingdom) with 1% glutamine, 40 μg of gentamicin sulfate (Garamycin; Schering Corp.) per ml, and 25% A$^+$ serum (The Bloodbank). Our monocyte monolayer contained 2×10^5 adherent cells per well (10a).

The different polysaccharides were dissolved in phosphate-buffered saline (PBS) and sterilely filtered through 0.2-μm-pore-size filters (Nuclepore). Stock solutions of LPSs from the different bacteria were dissolved in sterile water at a concentration of 1 mg/ml. The polysaccharide and LPS solutions were diluted in the medium described above and added to monocytes for 16 to 24 h before the supernatants were harvested.

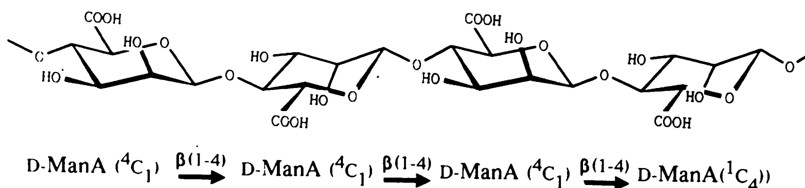
Flow cytometric quantification of binding of poly-M and LPS to monocytes. All steps for flow cytometric quantification were carried out at 0 to 4°C. Human monocytes on tissue culture plates were detached by use of a rubber policeman and washed once in ice-cold buffer containing 2.5% A$^+$ serum in PBS. The binding of poly-M to monocytes was accomplished by incubating 10^6 cells for 45 min with 5 μg of poly-M diluted in 100 μl of PBS-A$^+$ serum buffer. The cells were washed three times with this buffer before the addition of 100 μl of undiluted 2G8 hybridoma supernatant, which contains an immunoglobulin M monoclonal antibody (MAb) specific for poly-M. This MAb did not bind to G-blocks or LPS from *E. coli*, *Shigella flexneri*, *Salmonella minnesota*, *Vibrio cholerae*, *Klebsiella pneumoniae*, *P. aeruginosa*, or *S. minnesota* Re595 (Sigma) (12). After incubation for 30 to 40 min, the cells were washed three times with PBS-A$^+$ serum buffer before fluorescein isothiocyanate (FITC)-labelled goat-anti-mouse immunoglobulin (Becton Dickinson & Co.) was added for 35 min. The cells were then washed twice with this buffer and fixed in 2% formalin for 20 min (E. Merck AG, Darmstadt, Germany). Finally, the cells were washed once with this buffer, resuspended in PBS, and stored at 0 to 4°C until they were analyzed by use of a FACScan flow cytometer (Becton Dickinson). Blocking experiments were done by pretreating cells with 4,000 μg of G-blocks and 100 μg of LPS in 100 μl of PBS-A$^+$ serum buffer for 45 min before the addition of poly-M. Flow cytometric analysis was performed on a single-cell basis for 5,000 cells, and the data were displayed as frequency distribution histograms. The binding of LPS to monocytes was measured by adding 4 μg of FITC-LPS (Sigma) diluted in 100 μl of 10% A$^+$ serum in PBS to 10^6 cells for 45 min. The cells were washed three times with this buffer and thereafter treated as described above. Blocking experiments were done by pretreating cells with 200 μg of LPS, 500 μg of G-blocks, 500 μg of M-blocks, 200 μg of poly-M, and 200 μg of hyaluronic acid in 100 μl of PBS-A$^+$ serum buffer.

TNF-α assay. TNF-α activity was determined by measuring its cytotoxic effect on fibrosarcoma cell line WEHI 164 clone 13 as described earlier (11). Dilutions of recombinant human TNF-α (Genentech, South San Francisco, Calif.) were included as a standard. The TNF-α specificity of the assay was verified by use of a neutralizing MAb against recombinant human TNF-α (25). The results are presented as nanograms per milliliter ± the standard deviation for triplicate determinations.

IL-1 assay. IL-1 activity was determined by a two-stage assay. The first stage uses the mouse thymocyte EL-4 NOB-1 cell line, which produces high concentrations of IL-2 in response to human IL-1, as described by Gearing et al. (14). Dilutions of recombinant interleukin-1β (Glaxo, Geneva, Switzerland) were included as a standard. After incubation for 24 h, 100-μl quantities of the supernatants were transferred into a replicate 96-well microplate. The second stage of this assay uses the IL-2-dependent mouse T-cell line HT-2 as described by Mosmann et al. (30). A 100-μl HT-2 suspension (1.2×10^5 cells per ml) was added to each well and incubated for 24 h. IL-1 activity was completely neutralized by polyclonal antibodies against recombinant IL-1β and recombinant IL-1α (gifts from A. Shaw, Glaxo). The results are presented as nanograms per milliliter ± the standard deviation for triplicate determinations.

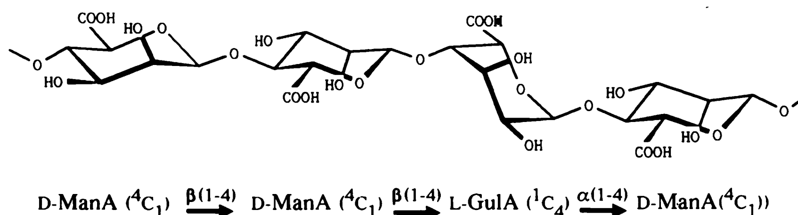
IL-6 assay. IL-6 activity was determined with IL-6-dependent mouse hybridoma cell line B.13.29 clone 9 as described by Aarden et al. (2). Dilutions of monocyte supernatants and recombinant IL-6 (6) as standards were incubated in a 96-well microplate together with cells (5×10^4/ml) for 72 h. The plate contents were harvested, and IL-6 activity was

Poly-M



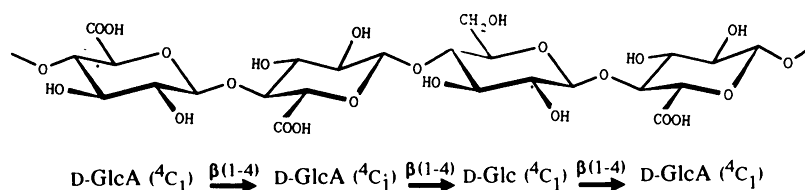
D-ManA ($^4C_1$) $\xrightarrow{\beta(1\text{-}4)}$ D-ManA ($^4C_1$) $\xrightarrow{\beta(1\text{-}4)}$ D-ManA ($^4C_1$) $\xrightarrow{\beta(1\text{-}4)}$ D-ManA($^1C_4$))

M-blocks



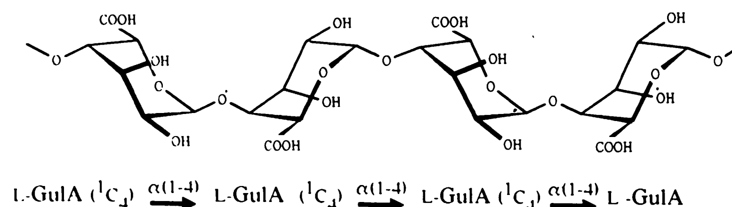
D-ManA ($^4C_1$) $\xrightarrow{\beta(1\text{-}4)}$ D-ManA ($^4C_1$) $\xrightarrow{\beta(1\text{-}4)}$ L-GulA ($^1C_4$) $\xrightarrow{\alpha(1\text{-}4)}$ D-ManA($^4C_1$))

C6OXY



D-GlcA ($^4C_1$) $\xrightarrow{\beta(1\text{-}4)}$ D-GlcA ($^4C_1$) $\xrightarrow{\beta(1\text{-}4)}$ D-Glc ($^4C_1$) $\xrightarrow{\beta(1\text{-}4)}$ D-GlcA ($^4C_1$)

G-blocks



L-GulA ($^1C_4$) $\xrightarrow{\alpha(1\text{-}4)}$ L-GulA ($^1C_4$) $\xrightarrow{\alpha(1\text{-}4)}$ L-GulA ($^1C_4$) $\xrightarrow{\alpha(1\text{-}4)}$ L -GulA

FIG. 1. Schematic representation of portions of the structures of the different polysaccharides used in this study.

determined colorimetrically (see below). IL-6 activity was completely neutralized by a polyclonal antibody against recombinant IL-6 (gift from W. Fiers, University of Ghent, Ghent, Belgium). The results are presented as nanograms per milliliter ± the standard deviation for triplicate determinations.

MTT assay. Viability in the assays for TNF-α, IL-1, and IL-6 was measured in a colorimetric assay for growth and survival by use of a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] as described by Mosmann (29). All data presented are from one representative experiment of at least three experiments.

RESULTS

Stimulation of monocytes by polysaccharides and LPS. In the first set of experiments, the TNF-α-inducing ability of β1-4-linked D-ManA polymers (poly-M) from *P. aeruginosa* was compared with that of β1-4-linked D-GlcA (cellulose oxidized in the C-6 position; C6OXY) and LPS from *E. coli*.

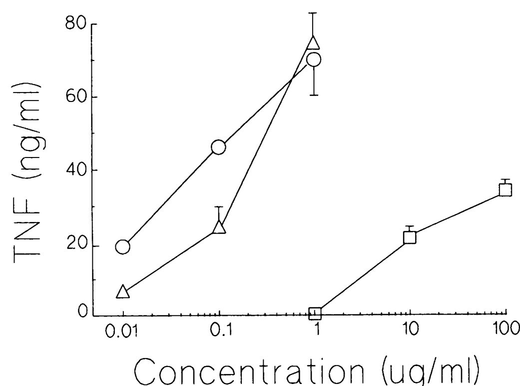


FIG. 2. Production of TNF-α from monocytes stimulated with poly-M (○), *E. coli* LPS (△), and C6OXY (□). The amount of TNF-α produced from unstimulated monocytes was less than 0.1 ng/ml.

Figure 2 shows that both polysaccharides tested were able to stimulate monocytes to produce TNF-α, although to various degrees. Figure 2 and Table 2 show that poly-M was approximately as efficient as LPS and about 500-fold more efficient than C6OXY. To exclude the possibility that the stimulatory effect of the polysaccharides was due to LPS contamination, we estimated the endotoxin contents of the various polysaccharides by the LAL assay. Thus, for C6OXY, the LAL assay indicated LPS contamination of less than 0.01% by weight. For poly-M, the LAL assay indicated an endotoxin content of less than 2%. Furthermore, as shown in Table 3, LPS isolated from *P. aeruginosa* was found to be about 100-fold less potent in inducing TNF-α production than LPS isolated from *E. coli*, and poly-M was a more potent TNF-α stimulator than LPS isolated from *P. aeruginosa*. As can be determined from Table 2, poly-M yields a flatter TNF-α dose-response curve than LPS from *E. coli*; thus, at concentrations below 0.1 μg/ml, poly-M is even more efficient than LPS in stimulating monocytes to produce TNF-α. Furthermore, as shown in Table 4, polymyxin B inhibited *E. coli* LPS-induced TNF-α production but had no effect on poly-M-induced TNF-α production in the same experiment. Taken together, these results suggest that the ability of poly-M from *P. aeruginosa* to stimulate monocytes to produce TNF-α is not due to contaminating LPS.

TABLE 2. TNF-α production from LPS- and poly-M-stimulated monocytes from different donors

Donor	Addition	TNF-α produced (ng/ml) when the addition was present at the following concn:		
		1 μg/ml	0.1 μg/ml	0.01 μg/ml
1	Poly-M	13.8 ± 1.2	12.7 ± 0.6	
	LPS	21.0 ± 2.9	14.6 ± 2.1	4.3 ± 0.2
2	Poly-M	41.1 ± 5.3	29.3 ± 3.8	23.5 ± 1.4
	LPS	47.3 ± 1.0	30.3 ± 3.6	22.6 ± 3.8
3	Poly-M		10.4 ± 1.1	3.4 ± 0.3
	LPS	38.3 ± 4.7	13.1 ± 0.7	3.4 ± 0.2
4	Poly-M	14.7 ± 1.8	19.5 ± 0.4	8.4 ± 1.6
	LPS	20.7 ± 3.4	13.6 ± 0.4	6.4 ± 0.9
5	Poly-M	70.2 ± 11.3	45.8 ± 1.7	18.7 ± 1.9
	LPS	74.2 ± 10.6	23.7 ± 3.9	6.4 ± 1.5
6	Poly-M	42.8 ± 15.0	56.3 ± 3.1	39.6 ± 4.8
	LPS	65.0 ± 36.3	31.7 ± 4.5	7.6 ± 0.2
7	Poly-M	4.6 ± 0.6	3.8 ± 0.1	2.7 ± 0.2
	LPS	17.7 ± 4.1	4.3 ± 0.8	0.9 ± 0.1
	Poly-M (mean ± SEM; *n* = 6 or 7)	31.2 ± 9.2	25.4 ± 6.8	16.0 ± 5.3
	LPS (mean ± SEM; *n* = 6 or 7)	40.6 ± 7.9	18.8 ± 3.5	7.4 ± 2.5

TABLE 3. TNF-α-inducing capacity of LPSs from *E. coli* and *P. aeruginosa* and poly-M from *P. aeruginosa* for human monocytes[a]

Addition	TNF-α produced (ng/ml) when the addition was present at the following concn:			
	1 μg/ml	0.1 μg/ml	0.01 μg/ml	0.001 μg/ml
E. coli LPS	17.7 ± 4.1	4.3 ± 0.8	0.9 ± 0.1	0.06 ± 0.02
P. aeruginosa LPS	1.1 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.10 ± 0.07
P. aeruginosa poly-M	4.6 ± 0.6	3.8 ± 0.1	2.7 ± 0.2	0.80 ± 0.09

[a] The amount of TNF-α produced from unstimulated monocytes was 0.13 ± 0.03 ng/ml.

Previously, it had been reported that for the β1-3-glucans to have optimal antitumor activity, their MWs must be higher than 50,000 (21). It was therefore of interest to examine the relationship between the MWs of β1-4-linked polyuronic acids and their capacity to induce TNF-α production. The results shown in Fig. 3 indicate that the stimulatory effect increased with increasing MWs of the polyuronic acids. Thus, the maximal stimulatory effect was obtained with average MWs higher than 50,000 and 200,000 for poly-M (≥99% β1-4-linked D-ManA) and high-M alginate (85% β1-4-linked D-ManA), respectively.

Inhibition of cytokine production by G-blocks. We previously reported that G-blocks (α1-4-linked L-GulA), in contrast to M-blocks (β1-4-linked D-ManA) and high-M alginate, do not stimulate monocytes to produce TNF-α, IL-1, or IL-6 (32). We therefore examined whether G-blocks could inhibit the cytokine-inducing capacity of M-blocks, poly-M, and C6OXY. As shown in Fig. 4, the addition of 1 mg of G-blocks per ml to different concentrations of M-blocks inhibited the production of TNF-α, IL-6, and IL-1 from monocytes. The degree of inhibition of cytokine production (percent relative decrease) was more than 97% at a 5:1 molar ratio of G-blocks to M-blocks (Fig. 4). G-blocks did not have

TABLE 4. Effects of polymyxin B on TNF-α production from monocytes stimulated with LPS from *E. coli* and poly-M from *P. aeruginosa*

Addition	TNF-α produced (ng/ml) in the absence (−) or presence (+) of polymyxin B at 50 μg/ml	
	−	+
Poly-M[a] (20 μg/ml)	5.9 ± 1.0	6.6 ± 2.3
LPS (1 μg/ml)	5.6 ± 0.4	0.7 ± 0.1
None (medium control)	<0.06	<0.06

[a] MW, 85,000.

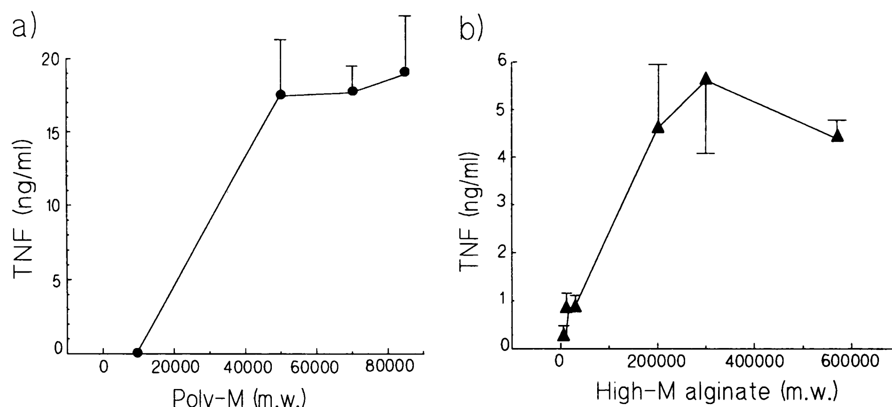


FIG. 3. Production of TNF-α from monocytes stimulated with poly-M (0.1 μg/ml) (a) and high-M alginate (10 μg/ml) (b) with different average MWs. The amounts of TNF-α produced from unstimulated monocytes were less than 0.08 ng/ml for the poly-M experiment and less than 0.16 ng/ml for the high-M alginate experiment.

any toxic effect on monocytes, as phorbol 12-myristate 13-acetate-induced TNF-α production was not affected by 1 mg of G-blocks per ml. Phorbol 12-myristate 13-acetate added by itself at 100 ng/ml induced TNF-α production of 6.4 ± 0.9 ng/ml and added with 1 mg of G-blocks per ml induced TNF-α production of 6.4 ± 0.6 ng/ml. We next examined the specificity of the inhibition of TNF-α production by G-blocks. Monocytes were stimulated with poly-M or C6OXY in the presence or absence of 1 mg of G-blocks per ml. As can be seen in Table 5, however, G-blocks inhibited to some degree the TNF-α production induced by both C6OXY and poly-M. The induced TNF-α production was inhibited by 58 or 60% for 10 μg of poly-M or C6OXY per ml, respectively. A similar degree of inhibition was seen with high-M alginate (data not shown).

LPS contains a polysaccharide portion, which has been suggested to play a role in the stimulation of TNF-α and IL-1 production (22, 27). Thus, we tested whether G-blocks could inhibit TNF-α production from LPS-stimulated monocytes. Figure 5a shows that the addition of 1 mg of G-blocks per ml to different concentrations of LPS indeed reduced the level of TNF-α production from monocytes. G-blocks inhibited LPS-induced TNF-α production in a dose-dependent manner, reaching 50% inhibition at 10 μg of G-blocks per ml and 90% at 1 mg of G-blocks per ml (Fig. 5b). It has been reported that the binding of LPS requires calcium, since ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) completely blocks the binding of LPS to hepatocytes (33). G-blocks have a strong ability to bind Ca^{2+} because of their configuration and charge (40). To ensure that the inhibition of LPS-induced TNF-α production by G-blocks when added together with LPS was not simply due to the removal of Ca^{2+} from the medium, we carried out the following experiment. Monocytes were washed three times in Hanks balanced salt solution (BSS) (GIBCO) and kept on ice. For experiments involving the addition of G-blocks, G-blocks in Hanks BSS (0.5 ml of a 2-mg/ml solution per well) were added, and the monocytes were incubated for 45 min. The monocytes were washed with cold Hanks BSS three times before the addition of different concentrations of LPS diluted in 25% A^+ serum in RPMI 1640 (complete medium, containing 0.33 mM Ca^{2+}) for 45 min at 0 to 4°C. Subsequently, the monocytes were washed three times with cold complete medium before the addition of 0.5 ml of complete medium per well and incubated for 16 to 24 h before the supernatants were harvested and analyzed for TNF-α. When LPS was present at 1 and 0.1 μg/ml and preincubation was done with Hanks BSS without 1 mg of G-blocks, TNF-α was produced at 9,500 ± 1,800 and 270 ± 40 pg/ml, respectively. Under the same conditions but with 1

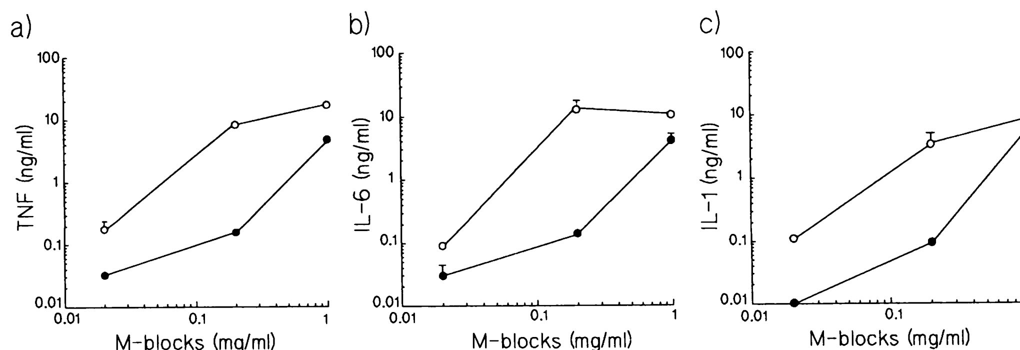


FIG. 4. Inhibition of TNF-α (a), IL-6 (b), and IL-1 (c) production from monocytes stimulated with M-blocks without (○) and with (●) the addition of 1 mg of G-blocks per ml. The amounts of cytokines produced from unstimulated monocytes were less than 0.03 ng/ml (TNF-α), 0.010 ng/ml (IL-6), and 0.015 ng/ml (IL-1). The amounts of cytokines produced from monocytes stimulated with 1 mg of G-blocks per ml were 0.170 ± 0.020 ng/ml (TNF-α), 0.030 ± 0.010 ng/ml (IL-6), and 0.020 ± 0.002 ng/ml (IL-1).

TABLE 5. Inhibition by G-blocks of TNF-α production from monocytes stimulated with poly-M and C6OXY[a]

Addition	TNF-α produced (ng/ml) when the addition[b] was present at the following concn:			
	100 μg/ml	10 μg/ml	1 μg/ml	0.1 μg/ml
Poly-M		15.4 ± 3.0	13.8 ± 1.2	12.7 ± 0.6
Poly-M + G-blocks (1 mg/ml)		6.4 ± 0.4	7.6 ± 0.9	7.2 ± 1.5
C6OXY	10.4 ± 1.4	8.1 ± 1.1	0.11	
C6OXY + G-blocks (1 mg/ml)	8.4 ± 1.3	3.2 ± 0.4	<0.08	

[a] The amount of TNF-α produced from unstimulated monocytes was less than 0.08 ng/ml. The amount of TNF-α produced from monocytes stimulated with 1 mg of G-blocks per ml was 0.09 ± 0.01 ng/ml.
[b] As indicated, the G-block concentration was 1 mg/ml.

mg of G-blocks, the corresponding values were 2,400 ± 200 and <78 pg/ml. Thus, preincubation with G-blocks inhibited TNF-α production from monocytes stimulated with LPS. The amount of TNF-α produced from unstimulated monocytes and monocytes preincubated with 1 mg of G-blocks per ml was less than 78 pg/ml. Furthermore, in a control experiment shown in Table 6, it was found that additional Ca^{2+} did not abolish the inhibition by G-blocks. Taken together, these data suggest that the inhibition obtained with G-blocks was not due to Ca^{2+} removal from the medium. The addition of G-blocks also inhibited TNF-α production from monocytes stimulated with LPSs from *P. aeruginosa*, *Vibrio cholerae*, *K. pneumoniae*, and *S. minnesota* (data not shown). These data indicate that G-blocks are able to inhibit the TNF-α-

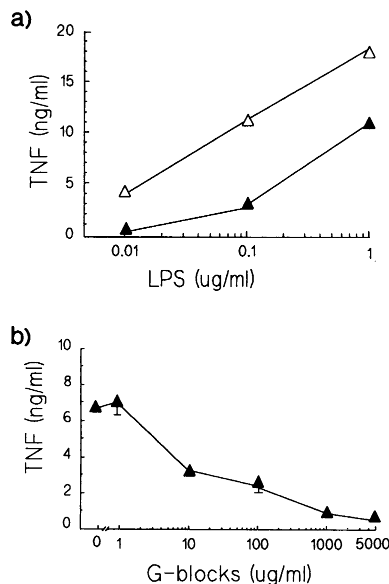


FIG. 5. (a) Inhibition of TNF-α production from monocytes stimulated with LPS in the absence (△) or presence (▲) of 1 mg of G-blocks per ml. The amount of TNF-α produced from unstimulated monocytes and monocytes stimulated with 1 mg of G-blocks per ml was less than 0.2 ng/ml. (b) Inhibition by increasing concentrations of G-blocks of TNF-α production from monocytes stimulated with 10 ng of LPS per ml. The amount of TNF-α produced from unstimulated monocytes and monocytes stimulated with various concentrations of G-blocks was less than 0.03 ng/ml.

TABLE 6. Inhibition of TNF-α production from LPS-stimulated monocytes by the addition of G-blocks and Ca^{2+}[a]

Concn (mM) of Ca^{2+} added	G-blocks[b]	TNF-α produced (pg/ml) when LPS was present at the following concn:	
		0.1 μg/ml	0.01 μg/ml
0.33	−	7,100 ± 300	1,700 ± 100
	+	3,200 ± 200	50 ± 10
0.66	−	8,100 ± 200	1,100 ± 100
	+	5,000 ± 600	50 ± 20
1.32	−	9,900 ± 1,000	1,300 ± 200
	+	4,200 ± 900	30 ± 10
2.64	−	9,200 ± 1,600	800 ± 90
	+	1,700 ± 200	10 ± 5
3.33	−	6,400 ± 700	630 ± 10
	+	1,800 ± 200	10 ± 5

[a] $CaCl_2 \cdot 2H_2O$ (1 M) was dissolved in sterile water, and the solution was filtered through 0.2-μm-pore-size filters. The Ca^{2+} solution was then diluted 1:100 in complete medium (containing 0.33 mM Ca^{2+}) before being added in different amounts to monocyte cultures in 16-mm wells. The volume in each well was 250 μl after the additions of Ca^{2+}, LPS, and G-blocks. The monocytes were incubated at 37°C for 16 to 24 h before the supernatants were harvested and analyzed for TNF-α. The amount of TNF-α produced from unstimulated monocytes and monocytes preincubated with 1 mg of G-blocks per ml was less than 3 pg/ml for all Ca^{2+} concentrations.
[b] Absent (−) or present (+) at 1 mg/ml.

stimulating activity of both β1-4-linked polyuronic acids and LPSs derived from several different bacteria.

Binding of polysaccharides and LPS to monocytes. Since G-blocks appeared to act as a competitive inhibitor of the induction by β1-4-linked polyuronic acids of TNF-α production, we examined whether G-blocks affected the binding of poly-M to monocytes. The binding of poly-M to monocytes was measured by use of flow cytometry and a MAb specific for poly-M (12). The histogram in Fig. 6 demonstrates that G-blocks inhibited the binding of poly-M to monocytes. Furthermore, Fig. 6 also shows that preincubation with LPS inhibited the binding of poly-M to monocytes. To further test whether LPS and the polyuronic acids recognize the same binding site, we measured the binding of FITC-LPS to monocytes in the presence and absence of the polyuronic acids. The histogram in Fig. 7 shows that the binding of

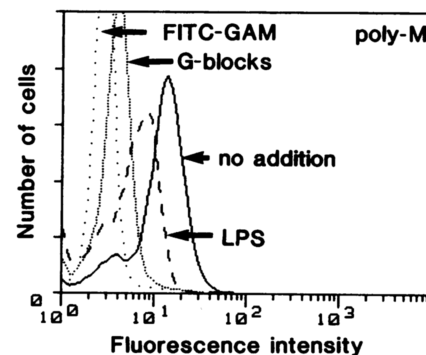


FIG. 6. Binding of poly-M (5 μg) to monocytes, as measured with MAb 2G8. Shown is the inhibition of binding of poly-M by pretreatment with G-blocks (4,000 μg) and LPS (100 μg). FITC-GAM, FITC-labelled goat anti-mouse immunoglobulin.

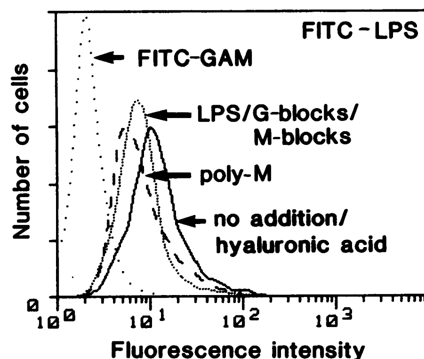


FIG. 7. Binding of FITC-LPS (4 μg) to monocytes. Shown is the inhibition of binding by pretreatment with LPS (200 μg), M-blocks (500 μg), G-blocks (500 μg), and poly-M (200 μg) but not by pretreatment with hyaluronic acid (200 μg). FITC-GAM, FITC-labelled goat anti-mouse immunoglobulin.

FITC-LPS was indeed inhibited by the addition of LPS, G-blocks, M-blocks, and poly-M but not by the addition of hyaluronic acid, a polysaccharide that is reported to bind to CD44 (24). Poly-M (200 μg) was a more efficient inhibitor of FITC-LPS binding than *E. coli* LPS (200 μg) and G-blocks (500 μg), suggesting common binding sites for the polyuronic acids and LPS.

Together, these data suggest that the polyuronic acids and LPS may compete for similar binding sites on monocytes. Furthermore, the concomitant inhibition by G-blocks of TNF-α production and binding suggests that these binding sites are important in the ability of the polyuronic acids and LPS to stimulate monocytes to produce TNF-α.

DISCUSSION

This report demonstrates that polymers of mannuronic acid are very potent stimulators of TNF-α production from human monocytes. The marked TNF-α-inducing ability of poly-M was not restricted to poly-M produced by *P. aeruginosa*, as similar activity was found for poly-M (94% D-ManA) isolated from liquid cultures of *Pseudomonas syringae* pv. phaseolicola (17; unpublished data). The bacterium *P. aeruginosa* is found in most patients with cystic fibrosis, and colonization is associated with pulmonary damage (35, 37). The facts that high-MW and very viscous polysaccharides containing large amounts of D-ManA are produced by several pathogenic *P. aeruginosa* serotypes (35, 37) and that polymers of D-ManA are very potent cytokine stimulators could partly explain the severity of this infection in humans. Our results are in agreement with previously reported data showing that mucoid exopolysaccharide isolated from *P. aeruginosa* induced splenic adherent cells (macrophages) to produce IL-1 (9).

The endotoxin content in poly-M was determined by the LAL assay to be less than 2%. The facts that poly-M is a more efficient stimulator per weight than LPS from *P. aeruginosa* (Table 3), that the addition of polymyxin B to poly-M did not reduce TNF-α production from monocytes (Table 4), and that no sugars other than D-ManA could be detected in a 400-MHz ^{1}H nuclear magnetic resonance spectrum of poly-M (>99% D-ManA) led to the conclusion that the high level of TNF-α production was due to polymers of β1-4-linked D-ManA and not to LPS contamination in the poly-M preparation.

Poly-M was found to be about 500-fold more efficient than C6OXY (Fig. 2 and Table 5). This difference in efficiency in inducing TNF-α production is probably related to the polysaccharide structure and not to the polymer length because the poly-M and C6OXY preparations used were in the same MW range (60,000 to 70,000). The three-dimensional structure of C6OXY (94% β1-4-linked D-GlcA) is very similar to that of poly-M (≥99% β1-4-linked D-ManA), except that the consecutive uronic acid residues in C6OXY are broken up by D-Glc (6%). The homopolymeric sequences of β1-4-linked uronic acids are consequently shorter in C6OXY than in poly-M, and it is possible that poly-M is more potent because of its content of longer homopolymeric β1-4-linked uronic acid sequences. The major difference between poly-M and high-M alginate (including M-blocks) is the length of the homopolymeric D-ManA sequences. High-M alginate and M-blocks contain about 15 and 5% L-GulA residues, respectively. The introduction of L-GulA into a homopolymeric D-ManA sequence changes the structure of the glycosidic linkages between the monomers in the polysaccharide. Thus, in high-M alginate and M-blocks, the D-ManA homopolymer is interrupted by L-GulA (4; Table 1 and Fig. 1). The maximal stimulatory effect of poly-M and high-M alginate was obtained at MWs higher than 50,000 and 200,000 respectively. High-M alginate was about 1,000-fold less efficient than poly-M, even at higher MWs (data not shown). Thus, our results suggest that both the length of the molecules and the length of the homopolymeric uronic acid sequences are important factors for the efficiency of polyuronic acids in stimulating monocytes to produce TNF-α.

In contrast to M-blocks, G-blocks do not stimulate cytokine production (32). Interestingly, the addition of G-blocks inhibited cytokine production from monocytes stimulated with high-M alginate (data not shown), M-blocks, C6OXY, poly-M, and LPS. The reduction by G-blocks of cytokine release was more pronounced at lower concentrations of M-blocks, high-M alginate (data not shown), C6OXY, and LPS, a result indicating competitive inhibition. The major structural difference between the β1-4-linked polyuronic acids and G-blocks (α1-4-linked L-GulA) is likely to be due to the different glycosidic linkages (Fig. 1). The chains in G-blocks are more compact than those in β1-4-linked polyuronic acids but still show some similarities in three-dimensional structure (4, 39). The inhibition of TNF-α production by G-blocks indicates that high-M alginate, M-blocks, poly-M, and C6OXY stimulate monocytes by similar mechanisms.

G-blocks also inhibited LPS-induced TNF-α production from monocytes (Fig. 5). One possible explanation for this effect is that the polysaccharide portion of LPS is involved in the stimulation of monocytes and that the polysaccharide portion bears structural similarities to G-blocks. The inner core polysaccharides in LPS show low levels of diversity compared with the O chain of the LPS molecule, and most of the known LPSs contain L-glycero-D-mannoheptose, a derivative of D-Man, and 3-keto-3-deoxy-D-mannooctulosonic acid linked to the lipid A portion (33, 34). Isolated polysaccharides of *Bordetella pertussis* endotoxin, as well as derived fragments containing the reducing 3-keto-3-deoxy-D-mannooctulosonic acid unit, are reported to be able to induce levels of IL-1 similar to those induced by native *B. pertussis* LPS (22). Furthermore, optimal TNF-α production requires complete S-form LPSs, and less active LPS derivatives with shorter polysaccharide chains are able to inhibit TNF-α production when added together with complete S-form LPSs (27). We found that G-blocks inhibited TNF-α

production from monocytes stimulated with LPSs from several different bacteria, including *P. aeruginosa*, *V. cholerae*, *K. pneumoniae*, and *S. minnesota*, as for *E. coli* LPS but with different efficiencies. Our results, together with these previously reported data, suggest that the polysaccharide portion of LPS may be involved in the stimulation of TNF-α production from monocytes, although the possibility that the inhibition of LPS activation by G-blocks is due to the masking of LPS receptors or effector molecules on the monocyte surface cannot be excluded.

The similarities between β1-4-linked polyuronic acids and LPS in the stimulation of monocytes to produce TNF-α are further supported by binding studies showing that the binding of poly-M to monocytes was inhibited by G-blocks and LPS and that the binding of LPS to monocytes was inhibited by poly-M, M-blocks, and G-blocks. Previous studies showed that LPS molecules containing different O-specific polysaccharides bind with different affinities to hepatocytes; LPS with an O unit containing only D-GlcA (as in C6OXY) binds with a higher affinity than the other LPS molecules tested (34). Furthermore, the inner core polysaccharide L-glycero-D-mannoheptose, a derivative of D-Man, is a very potent inhibitor of ^{125}I-LPS binding in this system (34). In addition, D-Man and yeast mannan, a polysaccharide rich in D-Man, inhibit the binding of seven different ^{125}I-LPSs to cells, even though three of these LPSs do not have D-Man in their polysaccharides (33). Furthermore, the binding of *B. pertussis* LPS to rat peritoneal macrophages is inhibited by the isolated O-specific polysaccharide from the same bacterium (18). These results correlate well with the hypothesis that the polysaccharide portion of LPS binds to a site that also recognizes polyuronic acids.

Four possible receptors or binding sites for LPS on the surface of monocytes or macrophages have been identified; they are the CD11-CD18 complex (41), a lectin-like binding site (18), CD14 (42), and an 80-kDa protein (23). The roles of CD18-CD11, CD14, and serum proteins such as LPS-binding protein and soluble CD14 in TNF-α production from monocytes stimulated with the different polysaccharides and LPS are currently under investigation in our laboratory.

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