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The Tumor Necrosis Factor-Inducing Potency of Lipopolysaccharide and Uronic Acid Polymers Is Increased when They Are Covalently Linked to Particles

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Lipopolysaccharide (LPS) and polymers of the uronic acid family stimulate monocytes to produce tumor necrosis factor (TNF). The TNF-inducing potency of these polysaccharides may depend on their supramolecular configuration. In this study detoxified LPS and uronic acid polymers have been covalently linked to particles which have been added to monocytes under serum-free conditions. Reducing the size of mannuronan from 350,000 to 5,500 Da (M-blocks) led to a 10- to 100-fold reduction in TNF-inducing potency. However, covalently linking the M-blocks to monodisperse suspensions of magnetic particles increased the TNF-inducing potency by up to 60,000-fold. Also, the TNF-inducing potency of glucuronic acid polymers was increased when they were linked to particles, but no potentiation was observed with guluronic acid blocks covalently attached to particles. Furthermore, O chains of LPS (detoxified LPS) became potent TNF inducers when they were presented to monocytes on a particle surface. No activation of the LPS-responsive SW480 adenocarcinoma cells was found with detoxified LPS or M-block particles, suggesting a preference for cells expressing CD14 and/or other membrane molecules. The potentiating effects were not restricted to polymers attached to aminated magnetic particles. Of particular interest, we found that short blocks of mannuronan induced TNF production also when covalently linked to biodegradable, bovine serum albumin particles.

Different uronic acid polymers with a β1-4 glycosidic linkage are able to stimulate monocytes to produce tumor necrosis factor (TNF) in a membrane CD14-dependent manner (6). Polymers of mannuronan [poly(M)] are the most potent of the β1-4-linked uronic acid polymers in inducing cytokine production (6, 27). The cytokine stimulatory activity of mannuronan is dependent of the molecular weight of the polymer, and optimal cytokine induction is obtained when the molecular weight is 20,000 or higher (27). Mannuronan and lipopolysaccharide (LPS) both stimulate monocytes to produce TNF by binding to membrane CD14 (6). In contrast to LPS, mannuronan does not stimulate U373 cells to produce interleukin 6 (IL-6), suggesting that the similarity in mechanisms of action between mannuronan and LPS is restricted to cells expressing membrane CD14 (6). The injection of mannuronan has been shown to protect mice from lethal X-irradiation, and this polymer also stimulates the generation of murine myeloid progenitor cells (12). Thus, mannuronan is a defined nonbranched polymer which activates parts of the innate immune system resulting in increased protection against various types of infections. Although there are no apparent toxic effects when mannuronan with a molecular weight higher than 100,000 is injected into mice (24a), it is important to use a polymer size as small as possible for therapeutic purposes.

The observation that optimal cytokine stimulation by mannuronan requires a certain polymer length may imply that enhanced effects can be obtained if the polymer has a certain supramolecular configuration which results in a multiple-receptor aggregation. Seljelid and coworkers found that β1-3-D-glucan has a higher level of biological activity in vivo when the polymer is linked to plastic microbeads (33). In addition, lipoteichoic acid from gram-positive bacteria induces enhanced TNF and IL-1β production when it is cross-linked on the monocyte membrane (24). LPS has been shown to exist in different supramolecular structures depending on the amount and distribution of the acyl chains in the lipid A region (34). When lipid A occurs in a cubic or inverted hexagonal structure, increased cytokine induction is observed, whereas a lamellar structure gives no cytokine induction (34). Although lipid A has been shown to induce many of the characteristic properties of LPS, the presence of 2-keto-3-deoxyoctonic acid sugars may potentiate the biological activity of LPS (16, 30). This underlines the importance of the sugar residues in LPS for cytokine-inducing potency.

In this study we investigated the effects of changing the supramolecular configuration of mannuronan and O-chain polysaccharides from LPS by covalently linking them to particles. The results show that the TNF-inducing potency of mannuronan as well as that of LPS is greatly enhanced by covalently linking them to particles.

MATERIALS AND METHODS

Polysaccharides. Poly(M) was isolated from agar colonies of *Pseudomonas aeruginosa* 8830, which was grown at 18°C as described previously (11). ^{14}C-labeled fructose (Amersham, Buckinghamshire, England) was added to the medium to make the alginate radioactive. The material was purified by a repeated combination of alkali treatment with 0.2 M NaOH at 45°C, precipitation with ethanol, and extraction of the precipitate by ethanol and chloroform. The polymer was dissolved in pyrogen-free water, filtered through a 0.22-μm-pore-size membrane filter (Millipore), and lyophilized. The content of mannuronic acid (ManA) in the polymer was estimated to be 92% by ^{1}H-nuclear magnetic resonance spectroscopy (9, 10), and the average molecular mass was estimated to be 350,000 g/mol by viscometry (Scott-Geräte). M-blocks (94% D-ManA) were prepared by hydrolysis of poly(M) for 1 h at 100°C and pH 5.6 and for 1 h at 100°C and pH 3.8. This procedure yielded M-blocks with an average molecular

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TABLE 1. Characteristics of the polyuronic acids used in this study

Polymer	Source	Mol wt	Monomer composition
Poly(M)	*P. aeruginosa*	350,000	92% D-ManA, 8% L-GulA
M-blocks	*P. aeruginosa*	<5,500	94% D-ManA, 6% L-GulA
G-blocks	*A. vinelandii*	5,500	94% D-GulA, 6% D-ManA
C6OXY	Cellulose	30,000	88% D-GlcA, 12% D-Glc

weight of 5,500 that were 94% D-ManA. For some experiments M-blocks with an average molecular weight of 3,000 were produced by additional hydrolysis.

G-blocks (94% L-guluronic acid [L-GulA]; degree of polymerization, 27) were isolated from colonies of *Azotobacter vinelandii* grown at 37°C with ^{14}C-labeled fructose (37).

C6OXY (β1-4-linked glucuronic acid [D-GlcA]) was prepared by the oxidation of cellulose at position C-6. The average molecular weight was estimated from intrinsic viscosity measurements to be 30,000, and the degree of oxidation (88% D-GlcA and 12% D-Glc) was determined by titration (25, 43). The characteristic features and structures of the uronic acids used in this study are summarized in Table 1 and Fig. 1. Endotoxin contamination in the different polysaccharides was measured by the *Limulus* amebocyte lysate assay (Chromogenix AB, Mölndal, Sweden). The estimated levels of endotoxin were as follows: M-blocks, 0.24 ng/mg; poly(M), 0.25 ng/mg; G-blocks, 12.4 ng/mg; C6OXY, 1.12 ng/mg.

LPS and detoxified LPS (D-LPS) from smooth *Salmonella minnesota* were purchased from Sigma. D-LPS was prepared by alkaline deacylation of LPS through the removal of the ester-linked fatty acids (3).

Covalent coupling of uronic acids and D-LPS to particles. The magnetic monodisperse suspensions of particles with epoxy groups (41) were aminated as described by Hermanson et al. (13). In some experiments hydrophilic bovine serum albumin (BSA; Sigma) particles were prepared according to the method described by Longo et al. (20). Uronic acids and D-LPS were coupled to magnetic monodisperse suspensions or BSA particles through the formation of amide bonds between the carboxylic groups on the uronic acids and the primary amine groups on the particles. The coupling was carried out in 0.1 M phosphate buffer, pH 7.3, by adding 1-ethyl-3-(3-dimethlaminpropyl) carbodiimide and *N*-hydroxysulfosuccinimide as described by Staros et al. (39). After linking oligo- and polysaccharides to the particles, the particles were extensively washed in 0.1 M phosphate buffer, pH 10, in order to remove noncovalently bound oligo- and polysaccharides. For some experiments particles of cross-linked BSA were made. The amounts of M- and G-block covalently linked to the particles were estimated by measuring the radioactivity with a beta counter (Packard). The characteristics of the particles used and the amounts of M-block and G-block coupled to them are given in Table 2.

Monocyte cultivation. Monocytes were isolated from type A$^+$ blood buffy coat (The Bloodbank, University Hospital, Trondheim, Norway) as described by Bøyum (1). Monolayers of monocytes in 24-well culture plates (Costar, Cambridge, Mass.) were cultured in AIM serum-free medium (Gibco Laboratories, Paisley, Scotland) with 1% glutamine and 40 μg of Garamycin per ml. Different concentrations of particles and oligo- and polysaccharides or LPS in solution were added to monocytes, and the supernatants were harvested 8 h later and assayed for TNF activity in the WEHI clone 13 bioassay (5).

SW480/β-gal cultivation. Human colon adenocarcinoma cells, SW480/β-gal cells (generously provided by Gerald Ranges, Miles Inc., West Haven, Conn.), contain a beta-galactosidase (β-Gal) gene under the control of the cytomegalovirus (CMV) immediate-early promoter/enhancer region (8). SW480/β-gal cells were grown in RPMI 1640 (Gibco Laboratories) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah), and 40 μg of Garamycin per ml (fetal calf serum medium). Stimulation with particulate and soluble forms of M-blocks and different forms of LPS was carried out in RPMI 1640 medium supplemented with glutamine, 20% human type A$^+$ serum (The Blood Bank), and Garamycin (A$^+$ medium). The β-Gal assay was performed essentially as described previously (18). Substrate conversion was measured as the optical density at 570 nm.

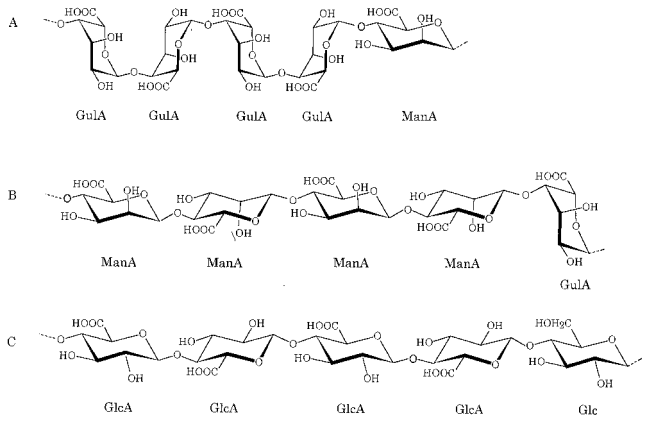

FIG. 1. Schematic representation of the structures of the uronic acid polymers used in this study. (A) G-blocks; (B) M-blocks; (C) C6OXY.

TABLE 2. Characteristics of the particles used in this study and the amount of covalently linked M-block and G-block

Particle type	ϕ[b] surface (μm/μm^2)	Concn of primary amino groups on the surface (mmol/g)	Amt (ng/10^6 particles) of:	
			M-block	G-block
J-205	4.5/ND[a]	0.65	33	ND
L-1172	4.2/2.3	0.11	12	17
R-409	4.5/3.8	0.50	50	47
BSA	5–10/ND	ND	120	ND

[a] ND, not determined.
[b] ϕ, particle number.

TNF assay. TNF activity was determined by measuring its cytotoxic effect on fibrosarcoma cell line WEHI 164 clone 13 as described previously (5). Dilutions of recombinant human TNF (generously provided by Refaat Shalaby, Genentech, South San Francisco, Calif.) were included as a standard. The TNF specificity of the assay was verified by use of a neutralizing monoclonal antibody against recombinant human TNF (19). The results are presented in units of picograms per milliliter ± standard deviations for triplicate determinations.

RESULTS

Induction of TNF from monocytes stimulated with ManA blocks covalently linked to monodisperse suspensions of polystyrene particles. We have previously found that soluble polymers of ManA stimulate monocytes to produce TNF through interaction with the CD14 receptor (6). Furthermore, poly(M) must be larger than 20 to 50 kDa in order to give optimal induction of TNF (27). In the first set of experiments we wanted to test if the presentation form of the polymer affected the TNF-inducing potency. Radiolabeled poly(M) with a molecular weight of 350,000 was degraded by acid hydrolysis to obtain M-blocks with a molecular weight of 5,500. As can be seen from Fig. 2A, reduction of the polymer size to 5,500 Da reduced the TNF-inducing potency by a factor of 10 to 100. However, covalently linking 5,500-Da M-blocks to R-409 and L-1172 particles resulted in 2,500- and 60,000-fold increases, respectively, in the TNF-inducing potency compared to that of soluble M-blocks (Fig. 2A). Linking M-blocks to particles also potentiated the TNF response compared to poly(M) in solution. Whether linking 5,500-Da G-blocks to L-1172 particles gave a similar potentiation of the TNF response was also tested. G-blocks in solution or linked to L-1172 particles did not induce the monocytes to produce TNF (Fig. 2B). Furthermore, replacing the amino groups on the particles with carboxyl groups did not enhance the TNF release from monocytes (data not shown). These data demonstrate that the stimulatory effect of M-blocks linked to particles is not caused by a net negative charge on the particles or a nonspecific reaction due to the coupling procedure.

Induction of TNF from monocytes with D-LPS covalently linked to particles. Previous studies have demonstrated that the TNF-inducing ability of LPS depends on the three-dimensional supramolecular structure (30). Since lipid A of the LPS molecule is responsible for the LPS structural conformation, it was of interest to examine the TNF-inducing activity of the polysaccharide part of LPS. In these experiments chromatographically purified D-LPS from *S. minnesota* delipidized by

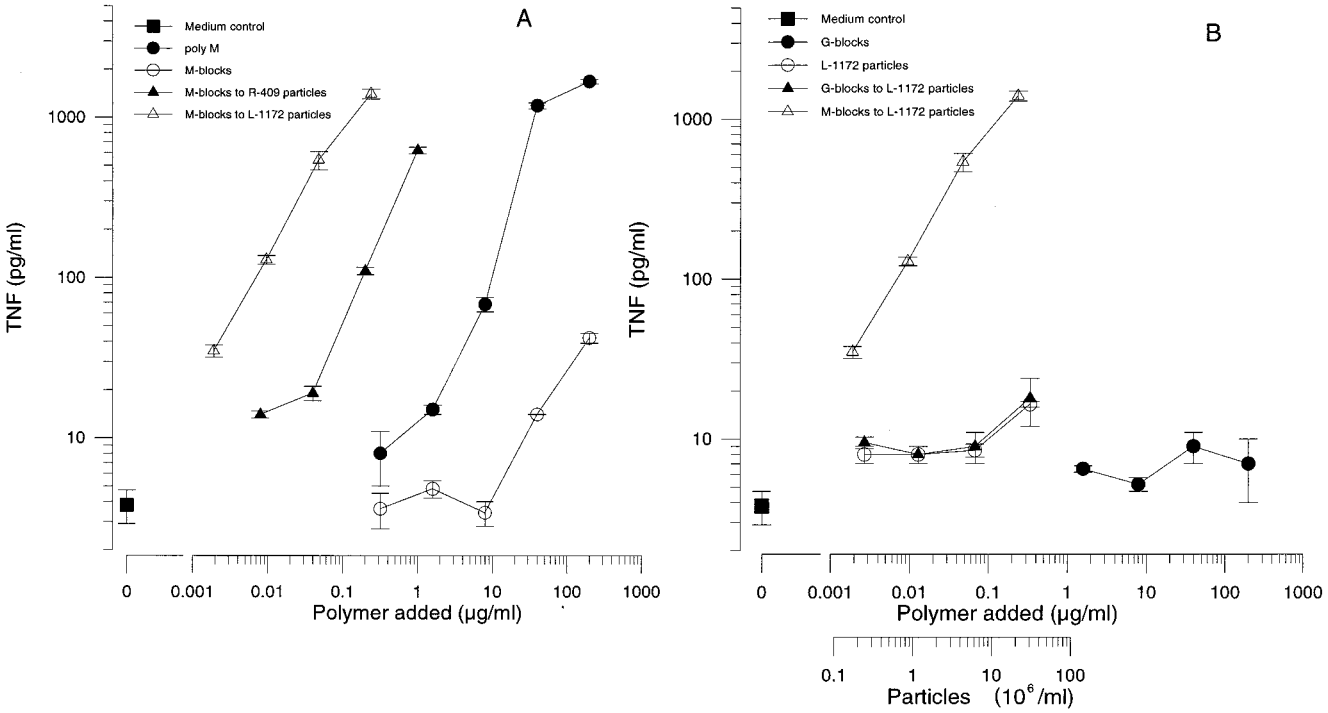

FIG. 2. (A) Effects of poly(M), M-blocks, M-blocks covalently linked to R-409 particles, and M-blocks covalently linked to L-1172 particles on TNF production from human monocytes. (B) Effects of G-blocks, G-blocks linked to L-1172 particles, M-blocks covalently linked to L-1172 particles, and L-1172 particles without polymer on TNF production. The stimulation of the monocytes was performed under serum-free conditions, and the level of spontaneous TNF release (medium control) is indicated. Similar data were obtained in three independent experiments.

alkaline hydrolysis was covalently linked to J-205 particles by the same method as that used for M-blocks. When D-LPS was tested on monocytes in solution under serum-free conditions it was found that concentrations of D-LPS up to 1 μg/ml did not induce monocytes to produce TNF, whereas LPS from *S. minnesota* 6261 gave a strong TNF response (Fig. 3A). When D-LPS was linked to particles and added to monocytes a high level of production of TNF, which was comparable to that with M-blocks linked to J-205 particles (Fig. 3B), resulted. The facts that the molecular weights of M-blocks and D-LPS are comparable and that D-LPS also was linked to the particles by amine bonds imply that the amount of bound D-LPS is equal to or less than the amount of M-block bound to the particles. Thus, these data suggest that polysaccharides from LPS are very potent TNF inducers when they are presented to monocytes on the surfaces of particles.

Activation of SW480/β-gal cells with D-LPS and M-block particles. The SW480/β-gal cells do not express functional membrane CD14 but respond well to LPS in the presence of serum (18). It was therefore of interest to determine if D-LPS or M-blocks, either in solution or linked to particles, could activate these cells. As can be seen from Fig. 4A, the complete LPS gave a strong and dose-related activation of the human CMV promoter in the SW480/β-gal cells, whereas D-LPS or M-blocks in solution had no stimulatory effect. In addition, M-block and D-LPS particles had no stimulatory effect on this cell type (Fig. 4B). These data indicate that M-block and D-LPS particles have a preference for stimulating membrane $CD14^+$ monocytes and not LPS-responsive cells which lack membrane CD14.

Induction of TNF from monocytes stimulated with GlcA particles. Another member of the uronic acid family, D-GlcA polymers, also stimulates monocytes to produce TNF in a CD14-dependent manner, although with less potency than that of poly(M) (6). In the next experiment cellulose was oxidized, which yielded a polymer consisting of 88% D-GlcA and 12% D-Glc with a molecular weight of 12,000. Adding this D-GlcA polymer to monocytes in solution resulted in a low level of production of TNF. However, linking the polymer to L-1172 particles resulted in a marked increase in the production of TNF (Fig. 5). The D-GlcA particles had approximately 10-times-less TNF-inducing potency than the M-block linked to L-1172 particles (Fig. 5). This result implies that the TNF-inducing effects of several different types of polysaccharides are potentiated when they are presented to monocytes on a particle surface.

Effects of M-blocks linked to BSA particles on TNF production. The ManA polymers may represent a new type of immunomodulators with interesting therapeutic potentials. If polymers are injected in vivo, it is beneficial to use a polymer with as low a molecular weight as possible. It was therefore considered important to test if M-blocks with a molecular weight around 3,000 stimulated monocytes to produce TNF when the polymer was covalently linked to biodegradable BSA particles. The results from this experiment are shown in Fig. 6. Adding soluble M-blocks to monocytes at a concentration up to 100 μg/ml did not result in the production of TNF. However, adding M-block–BSA particles to monocytes resulted in more than 1 ng of TNF per ml at a polymer concentration equivalent to 0.02 μg/ml (Fig. 6). This result demonstrates that biodegradable BSA particles can be used for potentiating the M-block effects on monocytes.

DISCUSSION

In this paper we have shown that the TNF-inducing potency of short ManA blocks can be greatly increased by covalently

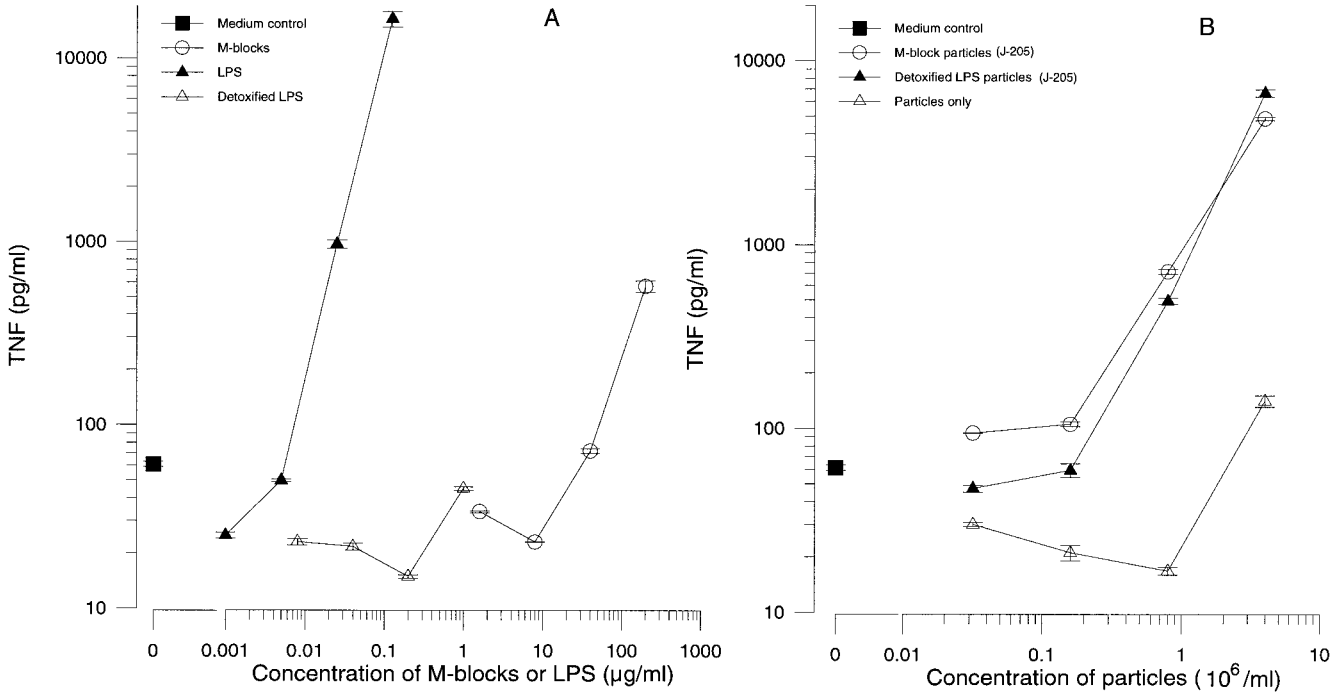

FIG. 3. (A) Effects of M-blocks, smooth LPS, and D-LPS on TNF production from human monocytes. The reagents were added in soluble forms. (B) Effects of M-blocks covalently linked to J-205 and D-LPS covalently linked to J-205 particles on TNF production from monocytes. J-205 particles without polymers (particles only) served as the control. The stimulation of the monocytes was performed under serum-free conditions, and the level of spontaneous TNF release (medium control) is indicated. Similar data were obtained in three independent experiments.

binding these polymers to particles. The TNF induction by M-block particles occurred under serum-free conditions, which rules out the contribution of the opsonizing effects of serum. Potentiation of the TNF production was also obtained by linking D-GlcA polymers to particles. However, no potentiation was observed when blocks of L-GulA were subjected to the same procedure, suggesting a requirement for uronic acid polymers with a β1-4 glycosidic linkage. The increase in the

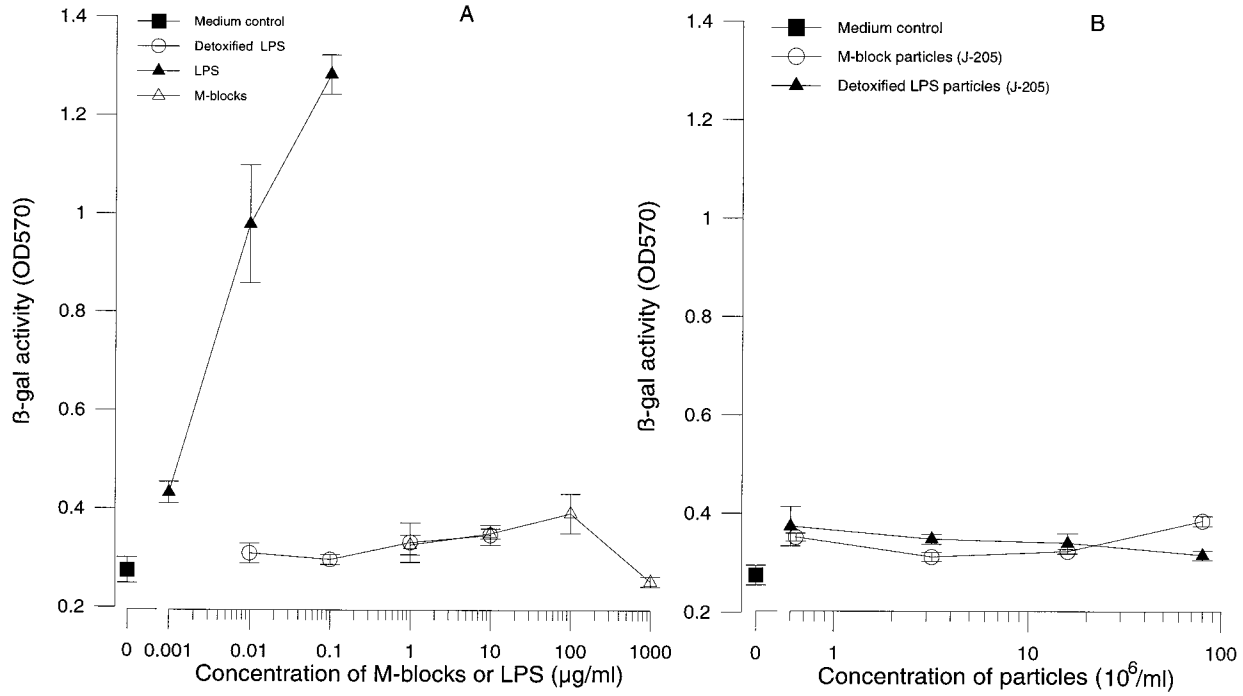

FIG. 4. (A) Effects of smooth LPS, D-LPS, and M-blocks on SW480 cells transfected with the β-Gal gene under the control of the CMV immediate-early promoter/enhancer region. The reagents were added in soluble form. (B) Effects of M-block and D-LPS covalently linked to J-205 particles. The β-Gal activity is presented as the optical density at 520 nm. Spontaneous β-Gal activity without stimulation (medium control) is indicated.

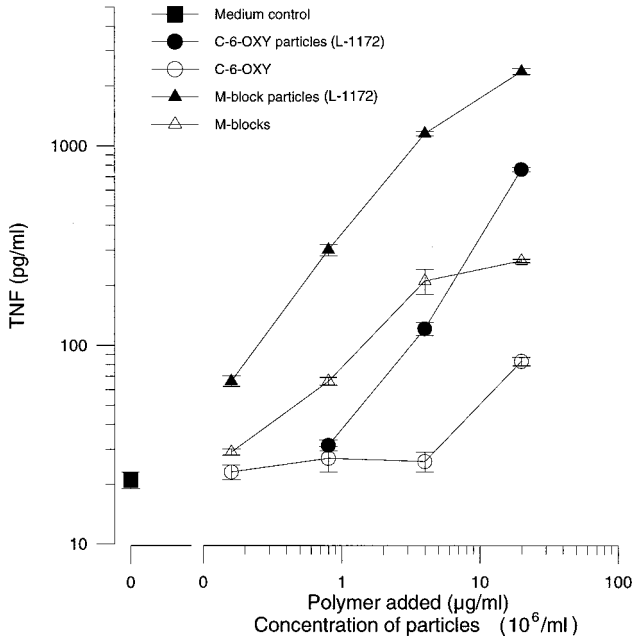

FIG. 5. Induction of TNF from monocytes stimulated with C6OXY (GlcA polymers) in solution (○), C6OXY on particles (●), M-blocks in solution (△), and M-blocks on particles (▲). The type of particle used in this experiments was L-1172. The spontaneous release of TNF (medium control) is indicated. Similar data were obtained in three independent experiments.

TNF-inducing potency was observed by linking M-blocks to different types of magnetic monodisperse suspensions of particles as well as to BSA particles. When particles with various amounts of M-block were compared for TNF-inducing potency, it was found that an increased amount of M-block on particles did not result in a higher level of TNF induction (Fig. 2A). This result may suggest that some degree of flexibility in the mannuronan on the particle surface is necessary for optimal activation of the monocytes.

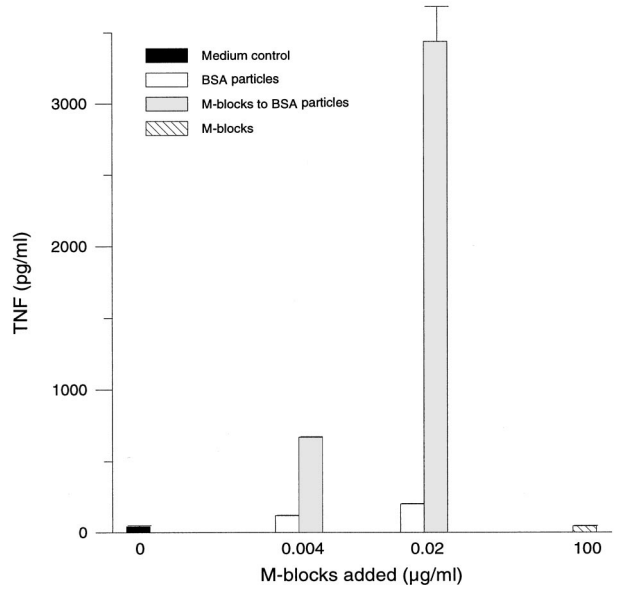

FIG. 6. Induction of TNF from monocytes stimulated with M-blocks in solution (▨), M-blocks linked to BSA particles (▭), and BSA particles without polymer (□). The spontaneous release of TNF (■) is indicated. Similar data were obtained in three independent experiments.

Removal of the fatty acids from LPS by mild alkali treatment results in reduced biological activity despite an intact polysaccharide portion (26). The endotoxic properties of LPS are generally associated with the lipid A region, but the 2-keto-3-deoxyoctonic polysaccharide of the inner core upregulates the lipid A activity (16, 30). It is not clear whether the action of LPS under physiological conditions is caused by aggregated or monomeric molecules since evidence for both models has been presented (35, 40). Soluble D-LPS at concentrations up to 1 μg/ml did not induce TNF production; however, covalently linking D-LPS to particles resulted in a pronounced TNF release, which reached the same levels as those obtained with M-block particles. This result implies that increased TNF production is obtained when O chains of LPS are presented to monocytes in an aggregated configuration such as on a particle or bacterial surface. Furthermore, lipid A may have an important role in potentiating the LPS activity by increasing the aggregated state of the molecule.

We have previously reported that mannuronan binds to CD14 on monocytes (6). After our initial observation several reports have now suggested a role for CD14 in responses to a variety of different compounds such as soluble peptidoglycan fragments and protein-free phenol extracts from *Staphylococcus aureus* (14, 17), rhamnose-glucose polymers from *Streptococcus mutans* (38), chitosans from arthropods (28), mycobacterial lipoarabinomannan (29, 31), and insoluble cell walls from different gram-positive bacteria (29). In addition, both membrane CD14 and soluble CD14 bind to the surfaces of gram-negative bacteria (15). Since mannuronan binds CD14, the addition of M-block particles to monocytes may result in the aggregation of CD14, with subsequent induction of TNF. Aggregation of cytokine receptors by receptor antibodies has been shown to induce biological effects in many receptor systems (4). Moreover, the aggregation of LFA-3, CD44, and CD45 with specific antibodies has been shown to induce TNF and IL-1β production in human monocytes (42). Also, some CD14 antibodies have been shown to induce the release of platelet activating factor as well as H_2O_2 production in monocytes (2, 21). The CD14 membrane protein has no transmembrane domain, which implies that CD14 by itself is not able to transduce a signal into the cell. Recently, Ingalls and Golenbock presented evidence that LPS can activate cells through CD11c/CD18 by using CHO cells transfected with this β2 integrin (14). Of particular interest is the recent data suggesting that CD14 may physically associate with CR3 (CD11b/CD18) in the presence of LPS (44). Several receptors for LPS in addition to CD14 exist (22). Furthermore, we have found that mannuronan particles stimulate monocytes through both CD14- and CD18-dependent mechanisms (6a). Thus, when M-blocks or D-LPS is present on a particle surface, multiple membrane receptors may be aggregated and this can be synergistic for the induction of TNF. The induction of TNF by M-block particles can be inhibited by dihydrocytochalasin B, suggesting that membrane contact over a large cell surface area is necessary for stimulation to occur (6a). Whether M-block or D-LPS particles induce CD14 to associate with members of the β2 integrin family or other signaling proteins is a question whose answer awaits further studies.

Despite the potent stimulatory activity of M-block and D-LPS particles on monocytes, no activation was observed on the LPS-responsive SW480/β-gal cells. No functional membrane LPS receptor is present on SW480/β-gal cells, and the LPS response on these cells requires soluble CD14 in serum (18).

Soluble CD14 has high affinity for LPS, and LPS-CD14 complexes are potent stimulators on several cell types which lack membrane CD14 (7). In contrast to LPS, soluble CD14 in serum is not sufficient to reconstitute the stimulatory activity of M-block or D-LPS particles on SW480/β-gal cells. This result may suggest that the ability of soluble CD14 to make stimulatory complexes with LPS requires an intact lipid A domain.

Carbohydrate-based immunomodulators have an interesting potential for the treatment of some cancer types as well as infectious diseases. Different forms of glucans, such as lentinan and Betafectin, have potent immunostimulatory activity and are now being tested in clinical trials (23). Also, glucans have been shown to stimulate macrophages and protect against lethal infections when linked to microbeads (32, 33). Mannuronan, which is structurally different from glucan, represents another carbohydrate immunomodulator with interesting immunostimulating properties (36). The molecular weight of mannuronan must be ≥20,000 in order to obtain optimal cytokine production (27) and protection against lethal *Escherichia coli* infection (3a). Despite the TNF-inducing properties of mannuronan in vitro, no apparent toxicity is observed when the polymer is injected into mice (24a). It is expected that the degradation and excretion of mannuronan is greater when the molecular weight of the polymer is low. Our data show that a mannuronan with a molecular weight of 3,000 stimulated monocytes to produce TNF when covalently linked to BSA particles. This result points to the possibility of using short blocks of mannuronan covalently linked to biodegradable particles as an immunomostimulator for the treatment of various types of infections.

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