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

GØRIL BERNTZEN,1 TRUDE H. FLO,1 ANDREI MEDVEDEV,1 LARS KILAAS,2 GUDMUND SKIÅK-BRÆK,3 ANDERS SUNDAN,1 AND TERJE ESPEVIK1*

1Institute of Cancer Research and Molecular Biology, 2SINTEF, Division of Applied Chemistry, and Institute of Biotechnology, 3The Norwegian University of Science and Technology, N-7005 Trondheim, Norway

Received 25 September 1997/Returned for modification 15 December 1997/Accepted 3 March 1998

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.
A. vinelandii M-blocks contain a β-galactosidase (assayed for TNF activity in the WEHI clone 13 bioassay (5)). They were added to monocytes, and the supernatants were harvested 8 h later and concentrations of particles and oligo- and polysaccharides or LPS in solution bridge, Mass.) were cultured in AIM serum-free medium (Gibco Laboratories, Mers used in this study. (A) G-blocks; (B) M-blocks; (C) C6OXY.

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 as the optical density at 570 nm. 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 deacetylation of LPS through the removal of the ester-linked fatty acids (3).

### 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 |

Poly(M) and G-blocks were prepared according to the method described by Hermanson et al. (13). In some experiments hydrophilic bovine serum albumin (BSA; Sigma) particles were prepared according to the method described by Staros et al. (39). After linking oligo-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 10, in order to remove noncovalently bound oligo- and polysaccharides. For some experiments particles of cross-linked BSA were made. The coupling was carried out in 0.1 M phosphate buffer, pH 7.3, by adding 1-ethyl-3-(3-dimethylaminopropyl) 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.

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

| Particle type | φb surface (μm²/μl) | Concn of primary amino groups on the surface (mmol/g) | Amt (ng/10⁶ particles) of |
|---------------|---------------------|---------------------------------------------------|--------------------------|
| J-205         | 4.5/ND              | 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                   |

*ND, not determined.

### 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. Radiolated 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 compared to poly(G) in solution was 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 virus (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.
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\( \mu \)g/ml did not induce monocytes to produce TNF, whereas LPS from \textit{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 \(\mu\)g/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 \(\mu\)g/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 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\( \mu \)g/ml did not induce monocytes to produce TNF, whereas LPS from \textit{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 \(\mu\)g/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 \(\mu\)g/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 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\( \mu \)g/ml did not induce monocytes to produce TNF, whereas LPS from \textit{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 \(\mu\)g/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 \(\mu\)g/ml (Fig. 6). This result demonstrates that biodegradable BSA particles can be used for potentiating the M-block effects on monocytes.
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. 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.

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.
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. 5). Similar data were obtained in three independent experiments.

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 these experiments was L-1172. The spontaneous release of TNF (medium control) is indicated. Similar data were obtained in three independent experiments.

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.
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/serum is not sufficient to reconstitute the stimulatory activity of CD14 and stimulate TNF-α-inducing ability of chitosans on monocytes: the involvement of CD14. FEBS Lett. 273:55–58.

5. Lynn, W. A., and D. T. Golenbock. 1992. Lipopolysaccharide antagonists. Immunol. Today 13:271–276.

6. Matsuoka, Y., and H. Yonekawa. 1995. Protective immunity for mannuronan C-5-epimerase activity. Carbohydr. Res. 276–287.

7. Seydel, U., K. Brandenburg, and E. T. Rietschel. 1981. Bacterial endotoxin: characterization of binding and tumor necrosis factor alpha induced. Infect. Immun. 61:1917–1925.

8. Seydel, U., K. M. Värnum, L. Ryán, and T. Espevik. 1994. Characterization of binding and TNF-α-inducing ability of chitosans on monocytes: the involvement of CD14. Vaccine 12:822–832.

9. Pugin, J., C. C. Schurer-Maly, D. Leturcq, A. Moriarty, R. J. Ulevitch, and P. S. Tobias. 1993. Lipopolysaccharide activation of human mononuclear and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc. Natl. Acad. Sci. USA 90:2744–2748.

10. Rietschel, E. T., U. Seydel, U. Zähringer, U. F. Schade, L. Brade, H. Lonn, W. Feist, M.-H. Wüthrich, M. Brandenburg, A. J. Umler, H.-D. Thal, K. Brandenburg, T. Kirikae, D. Grimes, and H. Brade. 1981. Bacterial endotoxin: molecular relationships between structure and activity, p. 753–779. In L.S. Young and M.P. Glauser (ed.), Infectious disease clinics of North America. W.B. Saunders Co., Philadelphia, Pa.

11. Savedra, R., Jr., R. L. Delude, R. R. Ingalls, M. J. Fenton, and D. T. Golenbock. 1996. Mycobacterial lipoarabinomannan recognition requires a lipooligosaccharide-beta1-3D polyglucose are strongly potentiated by con- junction to biodegradable microbeads. Scand. J. Immunol. 45:191–200.

12. Seljelid, R., Q. Gao, A. Berge, and J. Ugelstad. 1997. Biological effects of the immunomodulator beta 1-3D polyglucose are strongly potentiated by con- junction to biodegradable microbeads. Scand. J. Immunol. 45:191–200.
polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF-α release. J. Immunol. 154:851–860.
39. Staros, J. V., R. W. Wright, and D. M. Swingle. 1986. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. Anal. Biochem. 156:220–222.
40. Takayama, K., D. H. Mitchell, Z. Z. Din, P. Mukerjee, C. Li, and D. L. Coleman. 1994. Monomeric R lipopolysaccharide from Escherichia coli is more active than the aggregated form in the Limulus amebocyte lysate assay and in inducing Egr-1 mRNA in murine peritoneal macrophages. J. Biol. Chem. 269:2241–2244.
41. Ugelstad, J., A. Berge, T. Ellingsen, R. Schmid, T.-N. Nilsen, P. C. Mørk, P. Stenstad, E. Hornes, and Ø. Olsvik. 1992. Preparation and application of new monosized polymer particles. p. 87–161. In Progress in polymer science. Pergamon Press, Oxford, United Kingdom.
42. Webb, D. S., Y. Shimizu, G. A. Van Seventer, S. Shaw, and T. L. Gerrard. 1990. LFA-3, CD44, and CD45: physiologic triggers of human monocyte TNF and IL-1 release. Science 249:1295–1297.
43. Yackel, E. C., and W. O. Kenyon. 1942. The oxidation of cellulose by nitrogen dioxide. J. Am. Chem. Soc. 64:121–127.
44. Zarewych, D. M., A. L. Kindzelskii, R. F. Todd III, and H. R. Petty. 1996. LPS induces CD14 association with complement receptor type 3, which is reversed by neutrophil adhesion. J. Immunol. 156:430–433.