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Induction of Tumor Necrosis Factor Production from Monocytes Stimulated with Mannuronic Acid Polymers and Involvement of Lipopolysaccharide-Binding Protein, CD14, and Bactericidal/Permeability-Increasing Factor

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Well-defined polysaccharides, such as β1-4-linked D-mannuronic acid (poly[M]) derived from *Pseudomonas aeruginosa*, induce monocytes to produce tumor necrosis factor (TNF) through a pathway involving membrane CD14. In this study we have investigated the effects of soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), and bactericidal/permeability-increasing factor (BPI) on poly(M) binding to monocytes and induction of TNF production. We show that LBP increased the TNF production from monocytes stimulated with poly(M). Addition of sCD14 alone had only minor effects, but when it was added together with LBP, a rise in TNF production was seen. BPI was found to inhibit TNF production from monocytes stimulated with poly(M) in the presence of LBP, LBP-sCD14, or 10% human serum. Binding studies showed that poly(M) bound to LBP- and BPI-coated immunowells, while no significant binding of poly(M) to sCD14-coated wells in the absence of serum was observed. Binding of poly(M) to monocytes was also examined by flow cytometry, and it was shown that the addition of LBP or 10% human serum clearly increased the binding of poly(M) to monocytes. BPI inhibited the binding of poly(M) to monocytes in the presence of LBP, LBP-sCD14, or 10% human serum. Our data demonstrate a role for LBP, LBP-sCD14, and BPI in modulating TNF responses of defined polysaccharides.

Defined uronic acid polymers have been shown to induce cytokine production from human monocytes (25, 27). Of the uronic acid family, the β(1-4) mannuronan (poly[M]) is the most potent tumor necrosis factor (TNF) inducer (4, 25). Optimal cytokine stimulatory activity from uronic acid polymers requires a certain polymer length and mainly β(1-4) diequatorial glycosidical linkages (25). Uronic acids are common components of lipopolysaccharides (LPS) and extracellular polysaccharides from gram-negative bacteria and are also in the cell wall (as teichuronic acid) and extracellular polysaccharides of gram-positive bacteria (15). Large amounts of D-ManA are produced by several pathogenic *Pseudomonas aeruginosa* serotypes (28, 33). Uronic acid polymers and LPS both stimulate monocytes to produce TNF by binding to membrane CD14 (4, 37). In contrast to LPS, uronic acid polymers do not stimulate U373 cells to produce interleukin 6 (4), suggesting that the similarities in mechanisms of action between poly(M) and LPS are restricted to cells expressing CD14. Complex polysaccharides, such as β1-3-linked D-glucose with β1-6-D-glucopyranoside (Lentinan), are cytokine inducers and are reported to protect against neoplastic as well as infectious diseases (20). Poly(M) is a defined polymer with immunostimulating activities which may be of importance for treatment of infectious diseases.

Lipid A induces many of the characteristic properties of LPS; however, 2-keto-3-deoxyoctonic acid sugars potentiate its biological activity, which underlines the importance of the polysaccharide part for the immune-stimulating activities of LPS (14, 31). Serum or serum factors like soluble CD14 (sCD14), LPS-binding protein (LBP), and bactericidal/permeability-increasing factor (BPI) are able to interact with LPS and influence the LPS response, measured as cytokine production from monocytes (2, 11, 12, 14, 18, 22) or activation of other cell systems (5, 21, 30). We have previously shown that the ability of sCD14, LBP, and BPI to influence LPS-induced TNF production from monocytes depends on the LPS polysaccharide chain length (14).

In the present study we examined the involvement of serum, sCD14, LBP, and BPI on cytokine production from monocytes stimulated with poly(M) and on the binding of poly(M) to monocytes. The results clearly show that poly(M)-induced stimulation, as well as the binding of poly(M) to monocytes, is enhanced by LBP and inhibited by BPI.

MATERIALS AND METHODS

Reagents. An alginate highly enriched in mannuronic acid was isolated from the bacteria *P. aeruginosa* 8830, which was grown on agar plates at 18°C as previously described (9). 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. Finally, the material was subjected to gel filtration on Sepharose CL 4B. LPS contamination in poly(M) was measured by *Limulus* amoebocyte lysate assay (KABI Vitrum, Stockholm, Sweden). The level of endotoxin was <2.5 ng/mg of poly(M). No protein contamination could be detected in 1 mg of poly(M) by using the Bio-Rad protein assay (16). The content of mannuronic acid was determined to be 94% by ^{1}H nuclear magnetic resonance spectroscopy (7, 8), and the molecular weight was estimated to be 100,000 by viscometry (Scott-Geräte). In the binding studies of poly(M) to monocytes, a polymer with a molecular weight of 25,000 was used (4, 9). Recombinant sCD14 and LBP were prepared as described previously (6). Recombinant BPI was generously provided by M. Marra (Incyte, Palo Alto, Calif.). For binding studies of poly(M) to monocytes, a specific monoclonal antibody (MAb) called 2G8 was

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used (4). Heat-inactivated human A+ serum (HS) was obtained from The Bloodbank, University Hospital, Trondheim, Norway.

Monocyte cultures. Monocytes were isolated from human A+ buffy coat (The Bloodbank) as described by Bøyum (1). The isolated cells were seeded in 24-well culture plates (Costar, Cambridge, Mass.) and incubated for 90 min in 5% CO_2 at 37°C with RPMI 1640 medium (Gibco, Paisley, United Kingdom) including 1% glutamine and 40 μg of gentamicin per ml. The adherent mononuclear cell population was washed three times with Hanks balanced salt solution before serum-free AIM medium (Gibco) was added with or without HS, sCD14, LBP, or BPI, as described for each experiment. The cells were incubated for 6 to 7 h before the supernatants were harvested and assayed for TNF activity by the WEHI 164 clone 13 bioassay (3). Dilutions of human recombinant TNF (generously provided by R. Shalaby, Genentech, South San Francisco, Calif.) were included as a standard. The viability of the cells was measured in a colorimetric assay using a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as described by Mosmann (23). The specificity of the TNF assay was verified by adding TNF-neutralizing antibody 6H11 (19) to the monocyte supernatants. The results are presented in units of picograms per milliliter ± the standard deviation for triplicate determinations.

Flow cytometric quantification of poly(M) binding to monocytes. Binding of poly(M) to monocytes was done as previously described (4). Briefly, adherent monocytes were detached by a rubber policeman and washed three times in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, Mo.). Binding of poly(M) to monocytes was performed in the absence and in the presence of HS, LBP, sCD14, and BPI, as indicated in the figure legends. The cells were seeded in 96-well plates at a concentration of 10^6 cells/well with a final concentration of 100 μg of poly(M) per ml. The cells were incubated for 30 min at 0°C or 37°C, as indicated in the figures or figure legends, and then washed twice in 2.5% HS-PBS. All further steps were performed at 0°C. The cells were incubated with 50 μl of undiluted 2G8 hybridoma supernatant, which contains an immunoglobulin M (IgM) MAb specific for poly(M) (4). After two additional washes in 0.1% BSA-PBS, the cells were stained with 0.5 μl of fluorescein isothiocyanate-labeled goat anti-mouse Ig for 30 min. Finally, the cells were washed once, resuspended in PBS, and analyzed with a FACScan flow cytometer (Becton Dickinson, Lincoln Park, N.J.). Flow cytometric analysis was performed on a single-cell basis with 5,000 cells. The analysis results were displayed as frequency distribution histograms.

Binding of poly(M) to immunowells coated with BPI, LBP, or sCD14. Immunoplates (Nunc, Roskilde, Denmark) were coated with 50 μl of sCD14, LBP or BPI (each 5 μg/ml) overnight at 4°C. Nonspecific binding was blocked by 0.5% BSA-PBS before different concentrations of poly(M) diluted in 0.05% BSA-PBS were added for 40 min at 37°C. Poly(M) bound to the wells was detected by adding IIE10 hybridoma supernatant (50 μl/well) containing a specific poly(M) antibody of the IgM isotype. The generation of the IIE10 hybridoma was performed as described by Espevik et al. (4). No cross-reactivity between IIE10 MAb and lipid A or LPS from various sources was observed (data not shown). The immunowells were washed three times before peroxidase-labeled rabbit anti-mouse (Dakopatts, Glostrup, Denmark) antibodies were added. Bound antibodies were detected with *o*-phenylenediamine as a substrate (Dakopatts). The absorbance at 490 nm was measured with a microplate reader (Bio-Rad Laboratories).

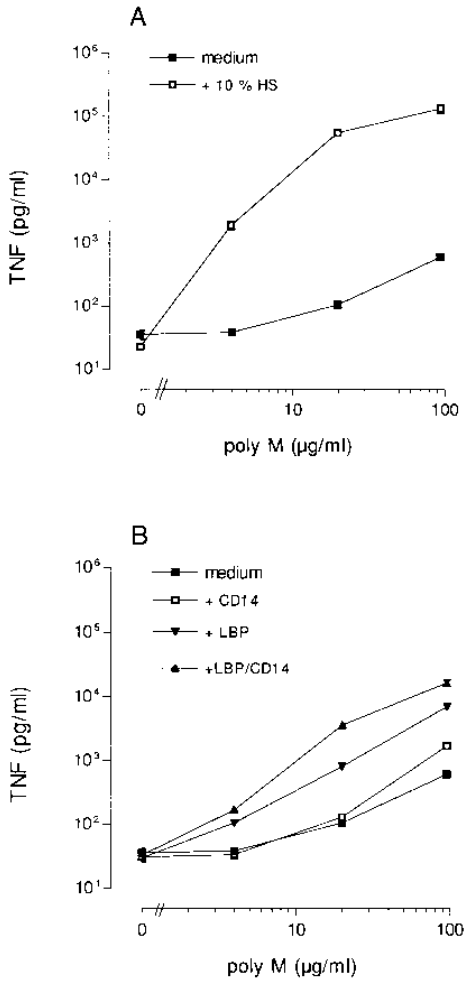

FIG. 1. (A) Production of TNF from monocytes stimulated with various concentrations of poly(M) in 10% HS or serum-free AIM medium; (B) effects of sCD14, LBP, and the combination of sCD14 and LBP on TNF production from monocytes stimulated with poly M. ■, AIM medium; □, 100 ng of sCD14 per ml; ▼, 100 ng of LBP per ml; ▲, 100 ng of sCD14 and 100 ng of LBP per ml. The standard deviations are within the symbols.

RESULTS

Previously, we have reported that poly(M) and other uronic acid polymers stimulate monocytes to produce TNF through the CD14 receptor (4). Furthermore, we have found that poly(M) binds to the CD14 on the monocyte cell membrane and that serum greatly upregulates the binding of poly(M) to the cells (4). It is not known what factors in serum modulate poly(M) stimulation and binding of poly(M) to monocytes. In this study we have investigated the effect of serum and serum factors on the potency of both the TNF-inducing ability of poly(M) and the binding of poly(M) to human monocytes.

In the first set of experiments, different concentrations of poly(M) were added to monocytes in the presence or in the absence of 10% heat-inactivated HS. As can be seen from Fig. 1A, serum enhanced poly(M)-induced TNF production 50- to 500-fold. LBP and sCD14 are serum proteins which potentiate LPS responses in monocytes (14, 18, 22). Human serum contains approximately 2 μg of sCD14 per ml (24) and <0.5 μg of LBP per ml (32). Thus, we added 100 ng of sCD14 per ml, 100 ng of LBP per ml, or combinations of the proteins to monocytes in the presence of various concentrations of poly(M). From the data presented in Fig. 1B, it can be seen that addition of LBP alone gave an approximately 10-fold increase in poly(M)-induced TNF production, while sCD14 had only a minor enhancing effect. Increasing the dose of LBP or sCD14 to 1 μg/ml did not further enhance poly(M) induction of TNF (data not shown). Combining LBP and sCD14 resulted in a greater increase in poly(M)-induced TNF production. These results suggest that LBP and sCD14 are important proteins in serum which potentiate poly(M) activities in monocytes.

BPI is a potent inhibitor of TNF production induced by smooth LPS, while rough LPS or lipid A is only marginally inhibited or not inhibited at all by BPI (14). This suggests that BPI may inhibit LPS effects by binding to the polysaccharide part of the LPS molecule. Thus, we wanted to examine the effect of BPI on poly(M)-induced release of TNF in the absence and in the presence of HS, LBP, and LBP-sCD14. The results show that BPI was able to inhibit poly(M)-induced TNF production only to a limited extent under serum-free conditions (Fig. 2A). However, in the presence of serum, BPI inhibited the TNF release by approximately 90% at a BPI concentration of 20 μg/ml and a poly(M) concentration of 4 μg/ml (Fig. 2B). A similar degree of inhibition by BPI was also ob-

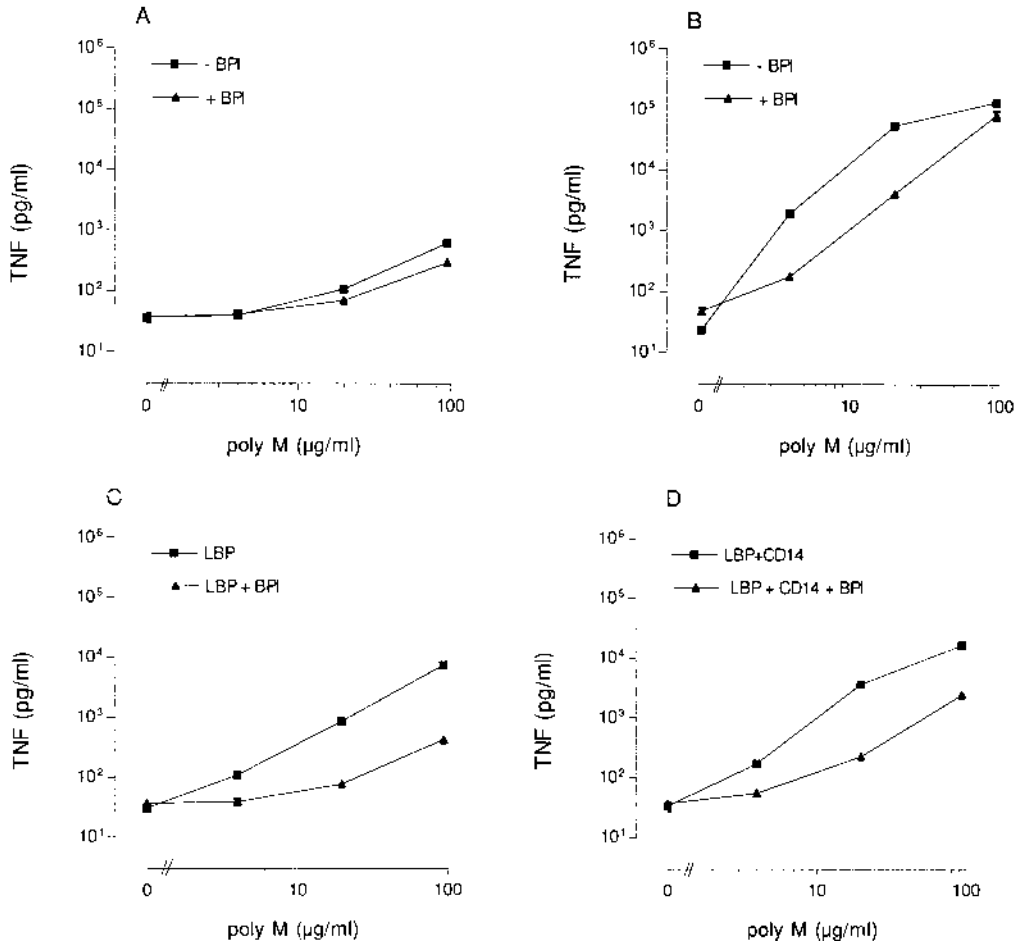

FIG. 2. Effects of 100 ng of BPI per ml on TNF production from monocytes stimulated with poly(M) in serum-free AIM medium (A) and 10% HS (B). (C) Effects of BPI on TNF production from monocytes stimulated with poly(M) in the presence of LBP. ■, 100 ng of LBP per ml in AIM medium; ▲, 100 ng of LBP and 100 ng of BPI per ml in AIM medium. (D) Effects of BPI on TNF production from monocytes stimulated with poly(M) in the presence of LBP and sCD14. ■, 100 ng of LBP and 100 ng of sCD14 per ml in AIM medium; ▲, 100 ng of LBP, 100 ng of sCD14, and 100 ng of BPI per ml in AIM medium. The standard deviations are within the symbols.

served when poly(M) was added together with LBP (Fig. 2C) or with a combination of LBP and sCD14 (Fig. 2D). From these data it is evident that BPI is a potent inhibitor of poly(M)-induced production of TNF, indicating that BPI may bind to poly(M) and thereby inhibit the enhancing effect of LBP.

This conclusion was further supported by experiments in which the binding of poly(M) to immunowells coated with BPI, LBP, sCD14, or BSA was estimated. Binding of poly(M) to coated immunowells was detected by a MAb against poly(M) which binds poly(M) and not LPS. It was found that poly(M) bound to BPI- and LBP-coated immunowells, while no significant binding to sCD14-coated wells was observed (Fig. 3). sCD14 was found to bind to the immunowells by using a CD14-specific antibody (data not shown). These data demonstrate that poly(M) is able to bind to both BPI and LBP.

Having established that LBP, sCD14, and BPI modulate poly(M)-induced TNF production, we next wanted to study the effects of these proteins on the binding of poly(M) to monocytes. We previously reported that poly(M) binds to CD14 on monocytes and that this binding is dependent on serum (4). In the first set of experiments, the binding of poly(M) to monocytes was estimated in the absence and in the presence of LBP, sCD14, and combinations of LBP and sCD14. The amount of poly(M) bound to the cells at 0 and 37°C was quantitated by flow cytometry and a poly(M)-specific antibody. In accordance with previously reported data (4), a low level of binding of poly(M) to monocytes occurred under serum-free conditions at 0°C (Fig. 4A). The addition of 1 μg of LBP per ml markedly enhanced the binding of poly(M) to monocytes both at 0 and 37°C (Fig. 4A, B). At 37°C a small increase in poly(M) binding could be observed both in the absence and in the presence of LBP (Fig. 4B). Reducing the LBP concentration to 0.1 μg/ml gave a similar enhancement of poly(M) binding to the monocytes (data not shown). By adding sCD14 a small increase in poly(M) binding could be observed at 37°C but not at 0°C (Fig. 4C, D). By combining LBP (1 μg/ml) and sCD14 (1 μg/ml), reduced binding of poly(M) compared to poly(M) binding with LBP alone was observed at 37°C, while little change in poly(M) binding occurred at 0°C (Fig. 4E, F). Thus, from this experiment one may conclude that LBP potentiates poly(M) effects by enhancing the binding of poly(M) to monocytes.

Since BPI is a potent inhibitor of poly(M) stimulation, we examined whether BPI affected poly(M) binding to monocytes. Under serum-free conditions BPI (10 μg/ml) only marginally inhibited poly(M) binding (Fig. 5A). However, in the presence of serum, LBP, or LBP-sCD14, BPI inhibited poly(M) binding almost to background levels (Fig. 5B to D). Reducing the BPI

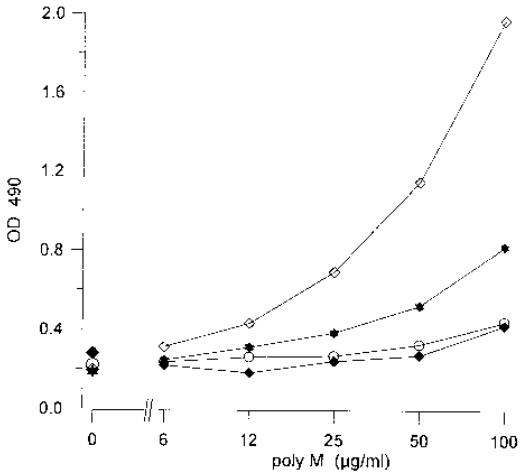

FIG. 3. Binding of poly(M) to immunowells coated with PBS (◆), sCD14 (○), LBP (*), or BPI (◇). Detection of poly(M) binding was done with the poly(M)-specific antibody IIE10.

concentration to 1 μg/ml gave a similar degree of inhibition of poly(M) binding to monocytes (data not shown). Control experiments showed that addition of BPI did not inhibit the binding of the poly(M)-detecting antibody (data not shown). These data demonstrate a clear correlation between BPI-induced inhibition of poly(M) stimulation and binding.

DISCUSSION

In this report we have shown that LBP, sCD14, and BPI modulate the TNF-inducing ability of poly(M) in monocytes as well as the binding of poly(M) to monocytes. Since poly(M) is isolated from gram-negative bacteria, it is of great importance to avoid LPS contamination. The poly(M) used in these experiments was therefore extensively purified by alkali treatment followed by extraction with ethanol and chloroform before gel filtration. This procedure yielded a polymer which contained less than 2.5 ng of LPS/mg of poly(M). The low level of endotoxin contamination is also supported by the fact that poly(M) does not stimulate the LPS-responsive, but membrane CD14-negative, U373 cell line to produce interleukin 6 (4). Also, polymyxin B does not inhibit poly(M)-induced TNF production (25). Other types of β1-4-linked uronic acid polymers from nonbacterial sources also stimulate monocytes but not U373 cells (4). In addition, binding of poly(M) to monocytes was detected with a poly(M)-specific antibody which does not recognize LPS (4). Thus, it is unlikely that LPS contamination accounts for the stimulatory activity of poly(M).

We have previously reported that poly(M) binds to CD14 on monocytes and that the binding occurs in the presence of serum (4). After this observation, several reports were published which identified a role for CD14 in the responses of cells to a variety of different compounds, such as soluble peptidoglycan fragments and protein-free phenol extracts from *Staphylococcus aureus* (17, 36), rhamnose-glucose polymers from *Streptococcus mutans* (34), chitosans from arthropods (26), mycobacterial lipoarabinomannan (29), and insoluble cell walls from different gram-positive bacteria (29). The ability of LBP to enhance the CD14-mediated responses of these compounds has not been addressed extensively. We found that LBP greatly enhanced poly(M)-induced TNF production and also the binding of poly(M) to monocytes. The CD14-dependent stimulation of peripheral blood mononuclear cells with extracts from *S. aureus* is not enhanced by LBP (17). Also, the CD14-dependent stimulation of mononuclear cells with soluble peptidoglycan from *S. aureus* is not affected by LBP (36). However, LBP enhances the TNF-inducing ability of soluble chitosans (26) and the stimulation of the monocyte cell line THP-1 by lipoarabinomannan (31a). LBP has been suggested to act as a lipid transfer protein which catalyzes the transfer of LPS to CD14 (10). According to Hailman et al. LBP does not make complexes with CD14 and LPS (10). This is in contrast to studies by Tobias et al. who found that LPS binds to LBP to form complexes (35). In the present study we found that poly(M) bound to LBP-coated immunowells, suggesting that LBP may interact directly with the polymer, which facilitates subsequent interaction with CD14 on the monocyte membrane. Our data imply that LBP may interact with defined polymers such as poly(M) in addition to LPS. Previously published data have shown that LBP is more potent for smooth than for rough LPS in stimulating TNF production from monocytes (14). This observation underlines the importance of the polysaccharide chain for the LBP effect.

The addition of sCD14 resulted in a small increase in

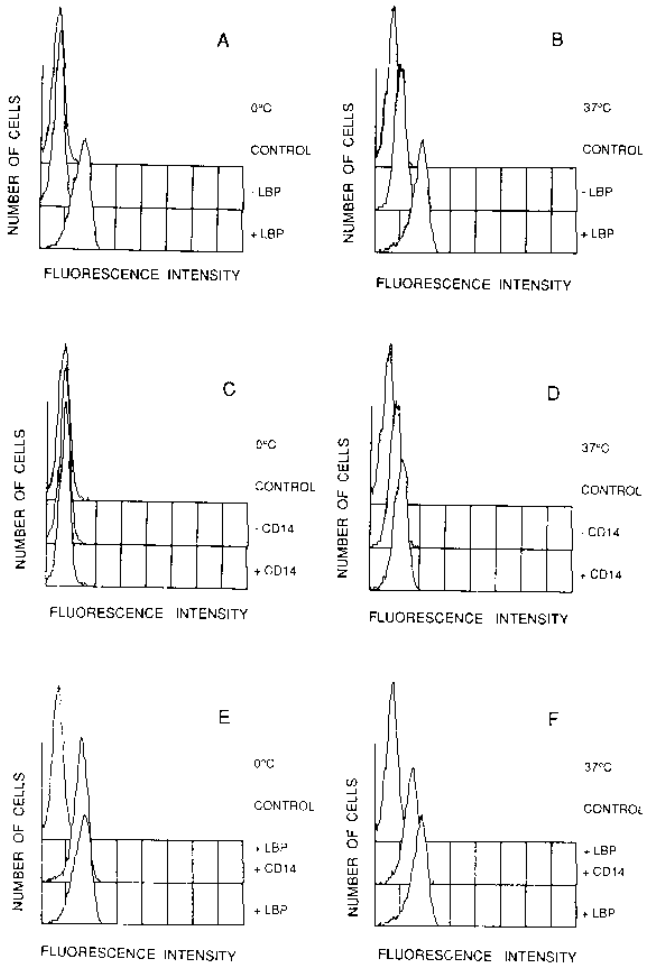

FIG. 4. Binding of poly M to monocytes in the presence of LBP (+ LBP), sCD14 (+ CD14), or a combination of LBP and sCD14 (+ LBP + CD14). Binding studies were performed at 0 and 37°C under serum-free conditions (0.1% BSA in PBS). LBP and sCD14 concentrations were 1 μg/ml. Control represents binding of fluorescein isothiocyanate goat anti-mouse Ig. Binding studies performed without LBP and sCD14 are indicated by − LBP, and − CD14, respectively.

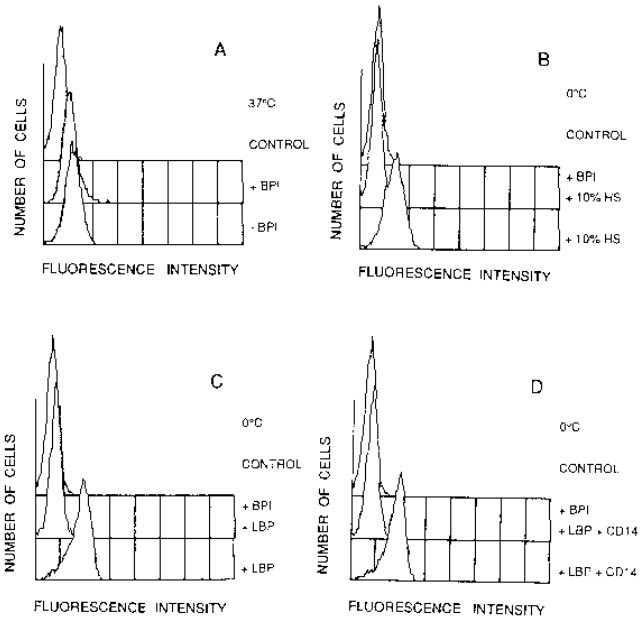

FIG. 5. Inhibition of poly(M) binding to monocytes by BPI. (A) Addition of 10 μg of BPI per ml in 0.1% BSA-PBS buffer (+ BPI) or no BPI addition (− BPI); (B) addition of 10 μg of BPI per ml in 10% HS-BSA buffer (+ BPI + 10% HS) or no BPI addition (+ 10% HS); (C) addition of 1 μg of LBP with (+ BPI + LBP) and without (+ LBP) 10 μg of BPI per ml in 0.1% BSA-PBS buffer; (D) addition of 0.1 μg of LBP and 0.1 μg of CD14 per ml with (+ BPI, + LBP + CD14) and without (+ LBP + CD14) 10 μg of BPI per ml in 0.1% BSA-PBS buffer. Control represents binding of fluorescein isothiocyanate goat anti-mouse Ig.

poly(M)-induced TNF production and also a small increase in the binding of poly(M) to monocytes at 37°C. Combining LBP and sCD14 further increased poly(M)-induced TNF production; however, it did not reach the level obtained with poly(M) in the presence of HS. This result suggests that unidentified serum proteins in addition to sCD14 and LBP may enhance the poly(M) response.

BPI and LBP are related proteins which contain binding sites for LPS. Responses induced by LPS are generally inhibited by BPI, which has been reported to bind to a site close to lipid A (6). The addition of BPI inhibited poly(M)-induced TNF production and also the binding of poly(M) to monocytes. Since poly(M) strongly bound to BPI-coated immunowells, it is likely that BPI inhibits binding of poly(M) to CD14, thus inhibiting binding of poly(M) to monocytes. Evidence for the existence of a binding site(s) for BPI on polysaccharides also comes from data showing that BPI has a higher neutralizing capacity for smooth LPS than for LPS which contains only 2-keto-3-deoxyoctonic acid sugars (14).

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 (13). Of particular interest are the recent data suggesting that CD14 may physically associate with CR3 (CD11b/CD18) in the presence of LPS (38). Whether poly(M) or LPS may induce CD14 to associate with CD11c/CD18 or other signaling proteins is a question that awaits further studies.

Defined polysaccharides which specifically stimulate the nonspecific immune system may be important agents for treatment of various infectious diseases. The mannuronic acid polymers may represent a new type of immunomodulators with interesting therapeutical potential. Injection of poly(M) has been shown to protect mice from lethal X irradiation, and poly(M) also stimulates the generation of murine myeloid progenitor cells (10a). Thus, poly(M) may activate the nonspecific immune system, resulting in increased protection against various types of infections.

ACKNOWLEDGMENTS

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