

Modulation of Lipopolysaccharide-Induced Monocyte Activation by Heparin-Binding Protein and Fucoidan

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Activated polymorphonuclear leukocytes release heparin-binding protein (HBP; also known as CAP37 or azurocidin) from azurophilic granules. HBP is a strong chemoattractant for monocytes that also prolongs monocyte survival and potentiates endotoxin (lipopolysaccharide [LPS])-induced production of tumor necrosis factor alpha (TNF- α). We investigated the binding of fluorescein isothiocyanate-conjugated HBP to human monocytes in the presence of EDTA and the polysaccharide fucoidan. EDTA, which chelates divalent cations, has been widely used to study the role of divalent cations in receptor-ligand interactions or enzyme activity. Fucoidan has been used to inhibit the binding of various ligands to scavenger receptors or selectins. Scavenger receptors are multiligand receptors that mediate endocytosis of proteases, protease-inhibitor complexes, lipoproteins, and LPS-lipid A. Fucoidan also interferes with leukocyte rolling by binding to L-selectins (expressed on leukocytes) and P-selectins (expressed on platelets and endothelium). We demonstrate that the binding of the neutrophil-derived protein HBP to monocytes is inhibited in the presence of EDTA and fucoidan. In addition, fucoidan and EDTA abrogate the enhancing effect of HBP on LPS-induced TNF- α production. These data provide supporting evidence that HBP binds to a receptor expressed on monocytes. This receptor is dependent on divalent cations and is possibly related to the scavenger receptor. Furthermore, we demonstrate that fucoidan, by itself, stimulates TNF- α release from isolated monocytes in a CD14-independent fashion. This is an important finding for the interpretation of results from studies that use fucoidan to “block” either scavenger receptors or L- or P-selectins.

Heparin-binding protein (HBP) is a 37-kDa protein that is released mainly from the azurophilic granules of neutrophils (15). Different molecular masses (29 to 37 kDa) for this molecule have been reported (15, 16, 36), probably due to different glycosylations of the protein. Also, the variable nomenclature of HBP reflects the involvement of different research groups in the identification of HBP. The protein was first isolated and purified from the granules of human neutrophils by Shafer et al. (36) and was named cationic antimicrobial protein (CAP37) because of its antimicrobial activity. Wilde et al. and Gabay et al. (16, 44) isolated a protein from the azurophilic granules of neutrophils and named it azurocidin. Subsequently, Flodgaard et al. (15) named the isolated protein heparin-binding protein because of its high affinity to heparin upon purification. The cloning of azurocidin cDNA resolved the controversy over the identity of CAP37, azurocidin, and HBP, with the conclusion that they were all the same molecule (1, 25).

The single-chain glycoprotein HBP bears many similarities to serine proteases, which are important in inflammatory processes (30). The greatest homologies found were to neutrophil elastase (47%), proteinase 3 (42%), and to a lesser extent to cathepsin G (37%). Even though HBP is a member of the serine protease family, it lacks protease activity due to mutations of two of the three amino acids in the highly conserved catalytic triad; that is, the histidine and serine residues are replaced by glutamine and tyrosine, respectively (15).

Despite the lack of proteolytic activity, HBP has a variety of physiological effects with a high potential for regulating monocyte function. Some of these monocyte-specific effects include

chemotaxis and increased longevity (29). We have demonstrated that HBP administered intraperitoneally increases monocyte recruitment into the peritoneum and increases survival in mice after peritonitis is induced by cecal ligation and puncture (23). Interestingly, HBP also enhances the production of proinflammatory cytokines (i.e., tumor necrosis factor alpha [TNF- α], interleukin-1 [IL-1], and IL-6) from isolated monocytes stimulated with endotoxin (lipopolysaccharide [LPS]) (32). In addition, HBP enhances the production of LPS-induced prostaglandin E₂ production from isolated monocytes (19).

In contrast to the intracellular release of many other neutrophil-derived antibiotic proteins, such as defensins or bactericidal permeability-increasing protein, 89% of HBP is released extracellularly (30). HBP, acting on monocytes, could therefore have a primary role in the influx of mononuclear cells in certain inflammations (29). Ostergaard et al. (27) first provided supporting evidence for a putative HBP receptor on monocytes. They determined that the saturated binding of HBP to monocytes was obtained at HBP concentrations of about 6 $\mu\text{g/ml}$ and a half-maximal binding was obtained at concentrations of about 1 $\mu\text{g/ml}$ (approximately 4×10^{-8} M). Subsequently, we have shown that HBP was internalized by monocytes, that HBP does not bind to CD14, and that HBP does not increase monocyte CD14 expression (19). In addition, the binding of labeled HBP was reduced in the presence of excess unlabeled HBP, again suggesting the presence of an HBP receptor.

Campbell (7) studied the binding of leukocyte elastase, cathepsin G, and lactoferrin to a receptor on human alveolar macrophages. It is noteworthy that two of these neutrophil-derived proteins (i.e., elastase and cathepsin G) are homologous to HBP. Campbell's results indicated a relatively low-affinity, high-volume receptor for this family of neutrophil

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granule glycoproteins. Binding of all three ligands was inhibited by the polysaccharide fucoidan. This observation led us to examine whether fucoidan also reduces the binding of HBP to monocytes. Fucoidan is a homopolymer of sulfated L-fucose that interferes with leukocyte rolling by binding to L-selectins and P-selectins (24, 37) but not to E-selectins (3). Fucoidan has also been used to inhibit the binding of various ligands to scavenger receptors (5, 12). Scavenger receptors are multiligand receptors that mediate endocytosis of proteases, protease-inhibitor complexes, lipoproteins (22, 40), and LPS-lipid A (17). We have shown by confocal microscopy studies that HBP is internalized by monocytes within 30 min. Furthermore, in this study, we have established that internalization of HBP is an important factor in the enhancing effect of HBP on LPS-induced TNF- α release from isolated monocytes (20), suggesting the possibility that the binding of HBP to monocytes could be inhibited with fucoidan.

Divalent cations such as Ca²⁺ or Mg²⁺ play an important role in the proper functioning of many receptors, e.g., the β_2 integrins on leukocytes (35), the mannose receptor on macrophages (43), and thrombospondin receptors on platelets (13). Interestingly, the same ligand may bind to cation-dependent and cation-independent receptors on cells, a phenomenon that depends on the activation status of the cell. For example, thrombospondin binds to a single receptor on nonactivated platelets in a cation-independent manner. However, upon platelet activation, thrombospondin binds to at least two other receptors that are both dependent on divalent cations (13). EDTA, which chelates divalent cations, has been widely used to study the role of Ca²⁺ or Mg²⁺ in receptor-ligand interactions or enzyme activity (4, 34). We therefore asked whether EDTA inhibits the binding of HBP to monocytes and whether EDTA inhibits the enhancing effect of HBP on LPS-induced TNF- α release from monocytes. This would suggest a cation-dependent receptor-ligand interaction.

In the present study, we used EDTA and fucoidan to characterize the HBP receptor on human monocytes. We show that the binding of fluorescein isothiocyanate (FITC)-conjugated HBP (FITC-HBP) to monocytes could be inhibited with fucoidan and EDTA. We also demonstrate that fucoidan abrogates the enhancing effect of HBP on LPS-induced TNF- α production (32). In addition, we show that fucoidan, by itself, induces TNF- α release from isolated human monocytes in a CD14-independent fashion.

MATERIALS AND METHODS

Reagents and monoclonal antibodies. Recombinant human HBP and FITC-conjugated HBP were a kind gift from Hans Flodgaard (Health Care Discovery, Novo Nordisk, Bagsvaerd, Denmark). Phosphate buffered-saline (PBS), bovine serum albumin, fucoidan, EDTA, and *Escherichia coli* O111:B4 LPS were purchased from Sigma Chemical Co. (St. Louis, Mo.). Phycoerythrin (PE)-coupled Mo2 (monoclonal mouse anti-human CD14 antibody; Coulter, Hialeah, Fla.) was used to tag monocytes. Purified mouse anti-human CD14 (MY4; 20 μ g/ml; Coulter) was used to block CD14, and isotype-matched immunoglobulin G2 (IgG2; Ancell, Bayport, Minn.) was used as a control in the antibody studies. An assay of the gelatin of *Limulus* amoebocyte lysate (sensitivity of 0.03 endotoxin unit; Associates of Cape Cod, Woods Hole, Mass.) was used to measure endotoxin levels. LPS concentrations in the fucoidan stock solution were between 0.6 and 1.2 ng/ml, and the calculated LPS concentrations in the samples were 18 to 36 pg/ml. To rule out that LPS contamination might be responsible for TNF- α production, we used END-X B15 beads (Associates of Cape Cod) to remove free LPS. END-X B15 beads have the potential to remove up to 1 μ g of LPS from the bulk liquid (14). We were able to reduce the initial LPS concentration in the fucoidan stock from 1.2 to <0.3 ng/ml. This resulted in a negligible final LPS concentration of <9 pg/ml in our samples. A previously established LPS dose response indicated that a threshold concentration of nearly 200 times that concentration (i.e., 1 to 2 ng of LPS per ml) was necessary to induce TNF- α from isolated monocytes in our experimental setting (19). An enzyme-linked immunosorbent assay (Biosource, Camarillo, Calif.) was used to measure TNF- α .

HBP affinity studies. Whole blood was collected from healthy volunteers and stored in acid-citrate-dextrose Vacutainers at room temperature. A dose- and time-response study for FITC-HBP affinity to CD14-positive monocytes was performed in initial experiments. In subsequent studies, blood was preincubated for 60 min with EDTA (1 to 30 mM final concentration), fucoidan (10 to 1,000 μ g/ml final concentration), or saline. FITC-HBP (10 μ g/ml final concentration) was then added, and the samples (100 μ l final volume) were incubated for another 60 min at 37°C with 5% CO₂. At the end of the incubation with FITC-HBP, monoclonal anti-CD14 antibody Mo2-PE (500 ng in 5 μ l) was added, and the samples were kept at 4°C for 25 min. Erythrocytes were removed by hypotonic lysis (150 mM ammonium chloride, 12 mM potassium bicarbonate, 0.1 mM EDTA), and the leukocytes were washed twice with FTA azide (Becton Dickinson, Cockeysville, Md.), fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

Fluorometry. Binding of FITC-HBP to monocytes was analyzed with a FAC-Scan from Becton Dickinson (Immunocytometry Systems, San Jose, Calif.) with an argon laser that emitted a beam at 488 nm. Fluorescence values derived from FITC-HBP were measured at 530 nm (FL1). CD14-positive monocytes were gated based on the combination of fluorescence derived from the anti-CD14 antibody Mo2-PE (measured at 580 nm; FL2) and the sideways scatter (35). A total of 4,000 to 5,000 CD14-positive monocytes were analyzed per sample, and acquired data were processed with Cellquest software version 1.2 (Becton Dickinson, Immunocytometry Systems). The FL1 fluorescence distribution (FITC-HBP) was displayed as a single histogram. The percentage of FL1 fluorescent cells and the mean fluorescence intensity were determined for each sample (see Fig. 1).

Since others have shown that acidic pH reduces fluorescence from FITC (26), we evaluated the possibility that changes in FL1 fluorescence were due to a direct effect of pH or EDTA on the FITC molecule. FITC was conjugated with bovine serum albumin, as previously described (2). FITC fluorescence was then measured with a spectrofluorometer (490-nm excitation wavelength, 535-nm emission wavelength; Aminco-Bowman, Silver Spring, Md.) at different pH values and in the presence or absence of EDTA (3 mM).

Monocyte isolation and culture. Human monocytes were isolated by dextran sedimentation and density gradient centrifugation (6). Briefly, whole blood was collected in EDTA Vacutainers, and one part of 6% dextran-500 in 0.9% (wt/vol) saline (Sigma Chemical Co.) was added to 10 parts of EDTA-blood. Leukocyte-rich plasma was harvested after 45 min of sedimentation and layered on top of 3 ml of 1-Step-Monocyte (1.068 gradient; Accurate, Westbury, N.Y.). The gradient was centrifuged at 600 \times g for 15 min at room temperature. The upper layer consisted of plasma and was discarded. The middle layer contained the monocytes and was harvested and washed twice with a washing solution containing 0.9% saline, 0.13% EDTA, and 1% fetal calf serum (BioWhittaker, Walkersville, Md.). The lower layer contained the remaining leukocytes and erythrocytes and was discarded. The monocyte cell suspension was centrifuged for 7 min at 600 \times g and resuspended in culture medium. The culture medium (RPMI 1640 with glutamine; Sigma Chemical Co.) was supplemented with 1% antibiotics (100 μ g of streptomycin per ml and 100 U of penicillin per ml; BioWhittaker) and 1% antimicrobials (0.25 μ g of amphotericin B per ml; Biowhittaker). The cells were counted with a hemocytometer, and the viability was assessed by trypan blue exclusion. In addition, the isolated cells were stained with Mo2-PE for analysis by flow cytometry of the percentage of CD14-positive monocytes. A total of 5 \times 10⁴ cells in 250 μ l of supplemented culture medium were added to each well (96-well plate; Costar, Cambridge, Mass.) and incubated at 37°C with 5% CO₂.

The isolated monocytes in the culture wells were pretreated for 60 min with either EDTA (10 mM final concentration) or fucoidan (0.3 mg/ml final concentration). They were then stimulated by the addition of saline (0.9%), LPS (10 ng/ml final concentration), HBP (10 μ g/ml final concentration), or a combination of LPS and HBP. The supernatant was collected after 24 h and analyzed for TNF- α by an enzyme-linked immunosorbent assay (Biosource). The TNF- α values for each donor were normalized according to the percentage of CD14-positive cells in the monocyte isolate.

Statistical analysis. Statistical significance was determined by analysis of variance (ANOVA) and Fisher's probable least-squares difference analysis (Statview 4.5; Abacus Concepts Inc., Berkeley, Calif.) (Fisher's post hoc test) to compare data between multiple groups. A *P* value of <0.05 was considered significant.

RESULTS

Affinity studies for FITC-HBP. Figure 1A demonstrates a dose-dependent affinity of FITC-HBP to monocytes. Figure 1B shows the specificity of the response as evidenced by a shift in FL1 fluorescence in CD14-positive monocytes relative to that of FITC-labeled IgG. An FITC-HBP concentration of 10 μ g/ml resulted in 100% fluorescent CD14-positive monocytes. These data suggest that monocytes express a receptor with affinity to HBP. In addition, the fact that HBP concentrations lower than 10 μ g/ml (i.e., 1 and 0.1 μ g/ml) did not enhance the LPS-induced TNF- α release from isolated monocytes (19) in-

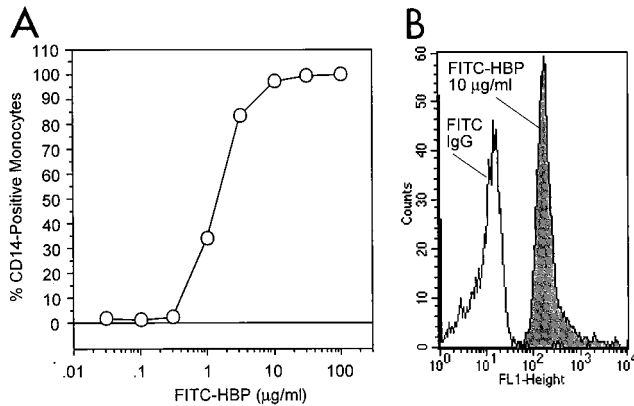


FIG. 1. Affinity of FITC-HBP to CD14-positive monocytes. Whole blood was incubated for 60 min with increasing concentrations of FITC-HBP (0 to 100 $\mu\text{g/ml}$) and stained with the monoclonal anti-CD14 antibody Mo2-PE, and CD14-positive monocytes were analyzed by flow cytometry as described in Materials and Methods. (A) Percentage of CD14-positive monocytes with FITC-HBP fluorescence; (B) a representative histogram showing FL1 fluorescence of control FITC-IgG and FITC-HBP (10 $\mu\text{g/ml}$).

indicates that these affinity studies reflect relevant binding of HBP to monocytes.

Effect of EDTA on FITC-HBP binding to CD14-positive monocytes. In the next set of experiments, we studied the requirement for divalent cations such as Ca^{2+} and Mg^{2+} in the binding of FITC-HBP to monocytes. Whole blood was incubated for 60 min with increasing concentrations of EDTA (final concentration, 1 to 30 mM) followed by a 60-min incubation with FITC-HBP (10 $\mu\text{g/ml}$). FITC-HBP fluorescence on CD14-positive monocytes was measured by flow cytometry. FITC-HBP fluorescence on monocytes was significantly reduced in the presence of 3 mM EDTA (Fig. 2A). For the saline-treated control group, the mean fluorescence intensity was 186 ± 10 (mean \pm standard error of the mean [SEM]), in comparison to 138 ± 5 for the 3 mM EDTA-treated group ($P < 0.0001$, ANOVA and Fisher's post hoc test). Higher

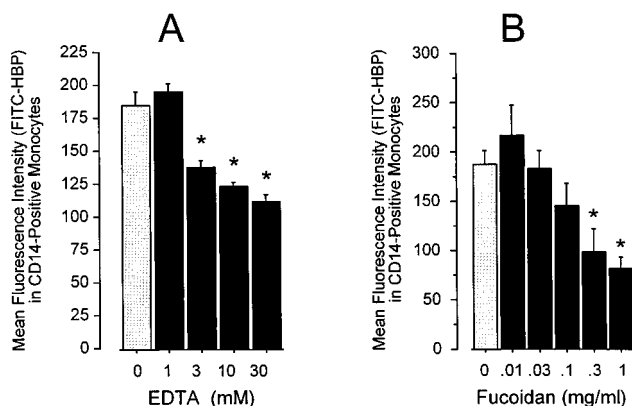


FIG. 2. Effect of EDTA and fucoidan on FITC-HBP affinity to CD14-positive monocytes. Whole blood was pretreated with increasing concentrations of EDTA (0 to 30 mM) (A) or fucoidan (0 to 1 mg/ml) (B) for 60 min and then incubated for an additional 60 min with FITC-labeled HBP (10 $\mu\text{g/ml}$). Samples were analyzed by flow cytometry. The results are shown as mean fluorescence intensity for FITC-HBP in CD14-positive monocytes. Values are means \pm SEMs ($n = 3$; measured in duplicate). *, $P < 0.05$ compared to the value for the saline control (gray bar) as assessed by ANOVA and Fisher's post hoc test.

TABLE 1. Effect of pH and EDTA on FITC fluorescence^a

Solution	Fluorescence intensity at pH of:			
	5	6	7	8
PBS	0.88	1.22	2.60	3.70
PBS + EDTA	1.57	1.71	3.72	3.55

^a FITC-labeled albumin was incubated in phosphate-buffered saline (PBS) or PBS plus EDTA (3 mM) at different pH values. Fluorescence intensity was measured at 535 nm with a spectrofluorometer and demonstrates a pH dependency, but EDTA had no effect on decreasing fluorescence intensity.

concentrations of EDTA produced slightly greater reductions in FITC-HBP binding.

It is known that FITC fluorescence is reduced in the presence of substances that decrease pH (26). Therefore, we tested the possibility of whether the reduction in FITC-HBP fluorescence in our binding studies (Fig. 2A) was due to a direct pH effect or an EDTA effect on FITC fluorescence. Table 1 demonstrates that EDTA by itself does not decrease and may even slightly increase FITC fluorescence. In addition, FITC fluorescence is reduced at low pH in both PBS and PBS-EDTA solutions.

Effect of fucoidan on FITC-HBP binding to CD14-positive monocytes. We tested the ability of fucoidan to reduce FITC-HBP affinity to monocytes because previous studies demonstrated that the binding of two neutrophil-derived proteins with homology to HBP (elastase and cathepsin G) was inhibited by fucoidan (7). Indeed, fucoidan produced a dose-dependent reduction in FITC-HBP fluorescence on CD14-positive monocytes starting at concentrations of 0.3 mg/ml (Fig. 2B). The largest concentration of fucoidan (1 mg/ml) produced a reduction in FITC-HBP fluorescence in monocytes of greater than 50%. At this concentration, the mean fluorescence intensity of FITC-HBP decreased from 187 ± 25 (mean \pm SEM) in the control group to 82 ± 19 in the fucoidan-treated group ($n = 3$; $P < 0.05$; ANOVA). Since fucoidan also competes with LPS to bind CD14 (9), we analyzed FL2 fluorescence derived from the anti-CD14 antibody Mo2-PE from these experiments. Pretreatment with fucoidan did not alter Mo2-PE binding (data not shown), suggesting that fucoidan does not quench Mo-2PE binding and does not increase CD14 expression.

Effect of EDTA on TNF- α production from isolated monocytes. The role of EDTA in the enhancing effect of HBP on LPS-induced TNF- α production (32) was assessed. LPS (10 ng/ml) significantly increased TNF- α production, and HBP (10 $\mu\text{g/ml}$) added to LPS significantly enhanced this effect (Fig. 3A, left panel). Pretreatment of monocytes with EDTA (10 mM) reduced TNF- α release from the LPS group and from the HBP-plus-LPS group (Fig. 3A, right panel). Although EDTA decreased TNF- α production levels in all groups, the production levels of TNF- α were still significantly increased after treatment with LPS or HBP plus LPS relative to those after treatment with saline or HBP alone. However, with EDTA pretreatment, there was no difference between levels for the LPS and the combination of HBP and LPS groups ($P = 0.103$, ANOVA and Fisher's post hoc test) (Fig. 3A).

Effect of fucoidan on TNF- α production from isolated monocytes. The effects of fucoidan on TNF- α production were evaluated in two separate sets of experiments. In the first set, fucoidan reduced the TNF- α production in the group treated with LPS (10 ng/ml) plus HBP (10 $\mu\text{g/ml}$) (from $2,175 \pm 317$ to $1,108 \pm 178$ pg/ml). However, TNF- α production in the fucoidan-treated group was elevated (968 ± 73 pg/ml) in com-

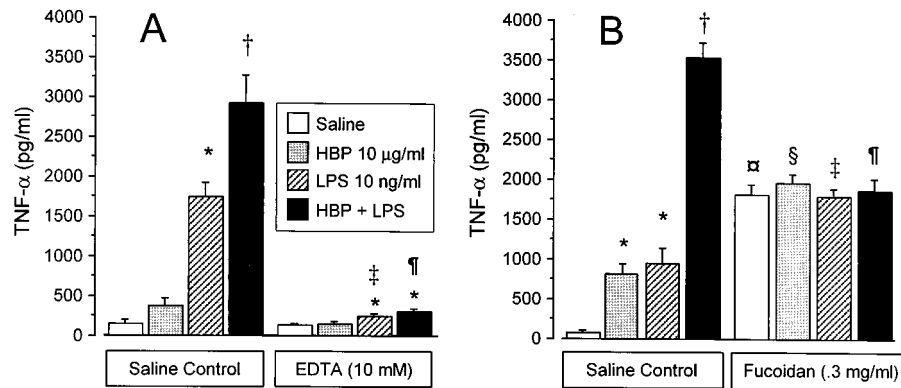


FIG. 3. Effect of EDTA and fucoidan on TNF- α production from isolated human monocytes. Isolated human monocytes were pretreated with EDTA (10 mM) (A), fucoidan (0.3 mg/ml) (B), or saline (A and B, saline control) for 60 min and then stimulated for 24 h with either saline, HBP (10 μ g/ml), LPS (10 ng/ml), or a combination of HBP plus LPS. Values are mean \pm SEMs ($n = 5$). Significant differences at a P of <0.05 were determined by ANOVA and Fisher's post hoc test. Symbols indicating significant differences within the saline-treated and EDTA- or fucoidan-treated groups: *, from saline and HBP; †, from saline, HBP, and LPS. Symbols indicating significant differences between the saline control-treated and EDTA- or fucoidan-treated groups: α , for saline; §, for HBP; ‡, for LPS; ¶, for HBP plus LPS.

parison to that in the saline-treated group (240 ± 40 pg/ml; $P = 0.0023$, ANOVA and Fisher's post hoc test).

In the second set of similar experiments, we evaluated whether the monocyte-activating effect of fucoidan was due to LPS contamination. END-X B15 beads were used to reduce LPS contamination levels to less than 9 pg/ml, which is nearly 200 times less LPS than the threshold concentration needed to induce TNF- α release (19). We evaluated the effect of fucoidan containing only these trace amounts of LPS on TNF- α release. The results are shown in Fig. 3B. Again, HBP enhanced the LPS-induced production of TNF- α (Fig. 3B, left panel, HBP + LPS). As demonstrated by the previous experiment, fucoidan abrogated the enhancing effect of HBP on LPS-stimulated TNF- α production (Fig. 3B, right panel, HBP + LPS) and induced TNF- α release by itself (Fig. 3B, right panel, saline).

Effect of CD14 blockade on TNF- α production from isolated monocytes. To ensure that fucoidan was not stimulating the LPS receptor, we blocked the receptor (CD14) with MY4 (20 μ g/ml) and then stimulated the monocytes with saline (0.9%), LPS (10 ng/ml), LPS (10 ng/ml) plus HBP (10 μ g/ml), or fucoidan (0.3 mg/ml). Preliminary experiments demonstrated that MY4 at a concentration of 10 μ g/ml effectively blocked CD14 over a 24-h period (data not shown). The results shown in Fig. 4, left panel, confirmed our previous findings (Fig. 3, left panels): LPS increased TNF- α production, HBP enhanced the LPS-induced TNF- α production, and fucoidan increased TNF- α production from isolated monocytes. Blockade of CD14 with MY4 (Fig. 4, right panel) abrogated the effect of LPS and also blocked the enhancing effect of HBP on LPS-induced TNF- α production. However, blockade of CD14 did not decrease the fucoidan-induced TNF- α production. The fucoidan-induced release level of TNF- α was $2,697 \pm 462$ pg/ml for the IgG control group, in comparison with $2,494 \pm 203$ pg/ml for the MY4 group.

DISCUSSION

We have established that both EDTA and fucoidan (Fig. 2) inhibit the binding of HBP to monocytes and also abrogate the enhancing effect of HBP on LPS-induced TNF- α production (Fig. 3). These results suggest that HBP binds to a receptor on monocytes that is dependent on divalent cations (e.g., Ca^{2+} and Mg^{2+}) and that this binding can be blocked with fucoidan.

Furthermore, we have demonstrated that fucoidan itself stimulates TNF- α release from isolated monocytes (Fig. 3B) and that this fucoidan-induced TNF- α release is CD14 independent (Fig. 4). These are novel findings and add a substance to the list of molecules that have the potential to release TNF- α from monocytes via CD14-independent pathways (10, 11, 31, 39).

The finding that EDTA partially reduces FITC-HBP fluorescence indicates that the putative HBP receptor requires divalent cations for optimal binding. However, the lack of complete inhibition (Fig. 2A) demonstrates that free divalent cations such as Ca^{2+} or Mg^{2+} are not an absolute requirement for FITC-HBP binding to monocytes or that there were still

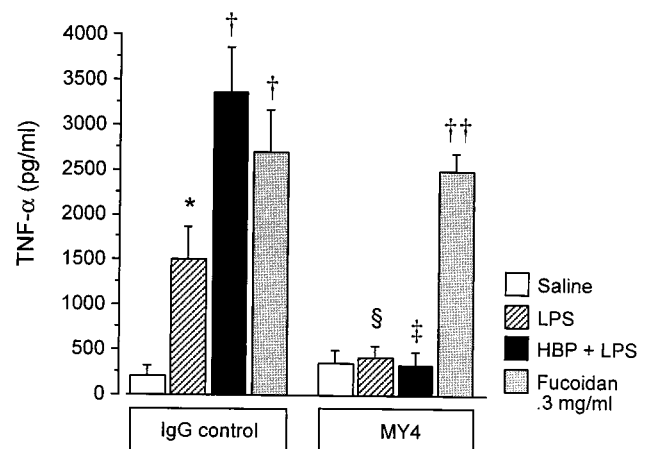


FIG. 4. Effect of CD14 blockade on LPS-, HBP-, and fucoidan-induced TNF- α production from isolated human monocytes. Isolated human monocytes were pretreated with the monoclonal anti-CD14 antibody MY4 (20 μ g/ml) or the IgG control for 60 min and then stimulated for 24 h with saline, LPS (10 ng/ml), a combination of HBP (10 μ g/ml) and LPS (10 ng/ml), or fucoidan (0.3 mg/ml). MY4 abrogated the inducing effect of LPS, and the enhancing effect of HBP on LPS induction, of TNF- α production, but MY4 did not affect the fucoidan-induced TNF- α production. Values are means \pm SEMs ($n = 5$). Significant differences at a P of <0.05 were determined by ANOVA and Fisher's post hoc test. Symbols indicating significant differences within the IgG control-treated and MY4-treated groups: *, from saline; †, from saline and LPS; ††, from saline, LPS, and HBP plus LPS. Symbols indicating significant differences between the IgG control-treated and MY4-treated groups: §, for LPS; ‡, for HBP plus LPS.

some free divalent cations in the solution. Free divalent cations also play an important role in LPS-induced TNF- α production. The presence of EDTA resulted in a nearly 90% reduction of LPS-induced TNF- α production from isolated monocytes (Fig. 3A). There was a similar reduction in TNF- α production when LPS and HBP were incubated together and there was no longer any enhancement of LPS effects by HBP (Fig. 3A). These results indicate that the presence of divalent cations is necessary for both the binding of HBP to its monocytic receptor and the effect of HBP to enhance LPS-induced TNF- α production.

Another important finding was that the polysaccharide fucoidan inhibited the binding of FITC-HBP to monocytes (Fig. 2B). Fucoidan has been used to inhibit receptor interaction for the class A scavenger receptor (22) and for P- and L-selectin (24, 37). Selectins are cell adhesion molecules expressed on leukocytes (L-selectins), platelets (P-selectin), and endothelial cells (E- and P-selectins). Selectins bind to carbohydrates and mucin-like structures and have an important role in the first (tethering-and-rolling) step of leukocyte recruitment to inflammatory sites (38). We believe that P-selectin is not a likely candidate as an HBP receptor, because P-selectin is expressed on endothelial cells and platelets but not on monocytes. Similarly, it is unlikely that L-selectin is an HBP receptor, because L-selectin is expressed on most leukocyte populations (8) but HBP has a higher affinity to monocytes than to granulocytes or lymphocytes (19).

An alternative HBP receptor candidate on monocytes could be a scavenger receptor. Fucoidan reduced the affinity of FITC-HBP to monocytes (Fig. 2B) and abrogated the effect of HBP to enhance LPS-induced TNF- α release from isolated monocytes (Fig. 3B). The class A scavenger receptor is an integral membrane protein that mediates endocytosis of modified lipoproteins (22, 28), and fucoidan has been widely used to inhibit this interaction (5, 12, 33). Importantly, we have demonstrated that HBP is internalized in monocytes by 30 min (19) and that internalization of HBP is essential for the effect of HBP to enhance LPS-induced TNF- α release from isolated monocytes (20). It is therefore appealing to speculate that the class A scavenger receptor, which mediates endocytosis (22), is a putative HBP receptor. Interestingly, the scavenger receptor on monocytes and macrophages is involved not only in the uptake of modified lipoproteins (22) but also in the uptake and detoxification of LPS-lipid A (17, 18). This suggests that scavenger receptors have a deactivating rather than an activating effect on monocytes/macrophages. Hampton et al. (17) demonstrated that concentrations of acetylated low-density lipoprotein that had bound the scavenger receptor did not induce TNF- α production in the macrophage cell line RAW 264.7. However, the authors did not evaluate a fucoidan-induced TNF- α production in human monocytes.

The LPS receptor CD14 is a glycosylphosphatidylinositol-anchored receptor (41) that lacks a transmembrane domain. CD14 is therefore unable to directly transduce cell signals. Hence, a transmembrane signaling molecule has been proposed (42), but to date, this proposed transducer molecule has not been identified and the very early signaling events of LPS are not fully understood. We have previously shown that HBP does not bind to the CD14 epitope recognized by the monoclonal antibody MY4 (19). However, the enhancing effect of HBP on LPS-induced TNF- α production is dependent on CD14, because this effect could be blocked by MY4 (Fig. 4). In other words, binding of LPS to CD14 is the key event, and HBP enhances this response in one (or more) signaling steps that follow this initial binding of LPS to CD14, either by an adjacent membrane effect or by an internal mechanism. It is

therefore possible that HBP and the putative HBP receptor enhance the LPS-induced signaling via a mechanism on the cell membrane that includes CD14. Interestingly, HBP possesses LPS binding activity (21) and may act like the LPS-binding protein LBP to direct LPS to CD14. Alternatively, it is possible that HBP does not elicit its effect on the cell membrane but amplifies the LPS-induced cell signaling after internalization. This hypothesis would also explain how MY4 abrogates the enhancing effect of HBP on LPS-induced TNF- α production (Fig. 4) without inhibiting HBP binding to CD14-positive cells (19). Indeed, studies from our laboratory have shown that internalization of HBP is an important step in enhancing effect of HBP on LPS-induced TNF- α production (20).

Fucoidan alone induced TNF- α production in a CD14-independent fashion, whereas HBP increased the LPS-induced TNF- α production in a CD14-dependent fashion (Fig. 4). Therefore, although HBP and fucoidan may bind to the same receptor, it is likely that the mechanism for fucoidan-induced TNF- α production is different from the mechanism that induces the effect HBP. The finding that LPS did not enhance TNF- α production after pretreatment with fucoidan indicates that fucoidan is not just an HBP analogue, again suggesting different activation pathways for fucoidan and HBP. It is possible that fucoidan changed the kinetics of TNF- α production so that the cells could not be stimulated by LPS. However, we do not have supporting data to confirm this hypothesis. Cavaillon et al. (9) demonstrated that CD14 has lectin-like properties, because binding of LPS to CD14 was inhibited by various polysaccharides (in order of inhibition effect, dextran sulfate > fucoidan > mannan > polygalacturonic acid > heparane sulfate > heparin > chondroitin sulfate). The binding activity was not correlated with the capacity to trigger TNF- α or IL-1, because dextran sulfate, which was a very efficient inhibitor of LPS binding, was not able to induce cytokine production. Conversely, fucoidan, which also competed with LPS for binding to the LPS receptor, triggered TNF- α and IL-6 production by isolated human monocytes (9). These results are consistent with our data. However, our experiments indicate a CD14-independent pathway for fucoidan-induced TNF- α production, whereas the experiments of Cavaillon et al. (9) showed a CD14-dependent pathway. We speculate that the higher fucoidan concentration used in our experiments (300 μ g/ml, compared with 20 μ g/ml in the experiments of Cavaillon et al.) might result in an alternative, CD14-independent pathway. Analysis of CD14 expression 90 min after fucoidan did not indicate a change in CD14 fluorescence, suggesting that fucoidan does not quench Mo2-PE binding and does not increase CD14 expression.

In summary, we have demonstrated that the binding of the neutrophil-derived protein HBP to monocytes is inhibited in the presence of EDTA and fucoidan and that fucoidan and EDTA abrogate the enhancing effect of HBP on LPS-induced TNF- α production. These data provide supporting evidence that HBP binds to a receptor expressed on monocytes. This receptor is dependent on divalent cations and is possibly related to the scavenger receptor. Furthermore, we have demonstrated that fucoidan, by itself, stimulates TNF- α release from isolated monocytes in a CD14-independent fashion. This is an important finding for the interpretation of results from studies that use fucoidan to block either scavenger receptors or L- or P-selectins.

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