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Heparin-Binding Protein (CAP37) Is Internalized in Monocytes and Increases LPS-Induced Monocyte Activation

Michael Heinzelmann, Mark A. Mercer-Jones, Hans Flodgaard, and Frederick N. Miller

Previous studies have shown that the neutrophil-derived heparin-binding protein (HBP), also known as CAP37 or azurocidin, potentiates the LPS-induced release of proinflammatory cytokines (TNF-α, IL-1, and IL-6) from isolated human monocytes. To date, the mechanisms by which HBP enhances LPS-induced monocyte activation have not been elucidated, and it is not known whether HBP also increases the LPS-induced production of other bioactive substances. We studied human monocytes activated by recombinant human HBP and LPS and their interaction with the LPS receptor CD14. We hypothesized that the stimulatory effect of HBP on the LPS-induced release of proinflammatory mediators from monocytes was mediated by specific binding of HBP to monocytes, which resulted in an up-regulation of CD14. Our results demonstrated that HBP alone (10 μg/ml) stimulated the production of TNF-α from isolated monocytes. In addition, HBP had an additive effect on LPS-induced production of TNF-α and PGE₂, suggesting a generalized monocyte activation. We used flow cytometry to demonstrate that HBP had a high affinity to monocytes but not to the LPS receptor CD14, and experiments performed at 4°C indicated an energy-dependent step in this process. Confocal microscopy showed that monocytes internalize HBP within 30 min. These data suggest that mechanisms other than increased CD14 expression are responsible for the enhanced release of TNF-α or PGE₂ in response to HBP and LPS.


Neutrophils are important effector cells in host defense that carry a variety of biologically potent substances in specialized granules (1). One of the main functions of the neutrophil is phagocytosis of pathogens during the early stages of an infection. Activated neutrophils release proteases, antibiotic proteins, and other granular contents into the maturating phagosome to eliminate phagocytosed microorganisms. Hence, neutrophils have traditionally been described as effector cells. However, neutrophils also have an important role in the afferent limb of inflammation (2). Neutrophils can synthesize and release cytokines, such as IL-1, TNF-α, and IL-6, and therefore modulate both T and B cell function. Another important role of neutrophils in the late stages of infection is the release of “classical” chemokines such as IL-8 (3) or other multifunctional proteins from azurophilic granules. There is increasing evidence that one such protein is a cationic antimicrobial protein with a molecular mass of 37 kDa (CAP37), which is also known as azurocidin or heparin-binding protein (HBP) (4).

Sequence analysis of HBP (5, 6) indicates that this protein bears many similarities to serine proteases, which are important in inflammatory processes (7, 8). The closest sequence homologies were found with neutrophil elastase (47%) and protease 3 (42%), also known as myeloblastin and p29. To a lesser extent, sequence homologies were found with cathepsin G (37%) and serine proteases from cytolytic T lymphocytes. However, 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—the histidine and serine residues have been changed to glutamine and tyrosine, respectively (9, 10).

Despite its lack of proteolytic activity, HBP has a variety of physiologic functions, and when HBP is released from neutrophils, it has a high potential for regulating monocyte function (4). In contrast to the intracellular release of many antibiotic proteins such as defensins or bactericidal permeability-increasing protein (BPI), 89% of neutrophil-derived HBP is released extracellularly during phagocytosis of Staphylococcus aureus (7). HBP could therefore be the molecule responsible for the second wave of mononuclear cells in certain inflammations (4). HBP is a multifunctional protein with specific and powerful chemotactic properties for monocytes (7). Furthermore, HBP not only attracts monocytes but also increases monocyte survival (11), activates monocytes to secrete thrombospordin (11), and increases LPS-induced monocyte production of the proinflammatory cytokines TNF-α, IL-1, and IL-6 (12). In addition, HBP has antimicrobial activities at pH values found in maturing phagosomes (13). The bactericidal site of action appears to localize to the inner membrane of Gram-negative bacteria, and initial events involve HBP binding to the lipid A moiety of LPS (14, 15).

Clearly, the membrane-bound CD14 receptor plays a major role in LPS-mediated monocyte activation (16). However, other LPS receptors such as the β-2 integrin subunit CD18, which is involved in the nonsonic recognition of LPS, or an acetyl lower density lipoprotein receptor on monocytes, which is involved in the uptake and detoxification of LPS/lipid A, may also play a role in monocyte activation (17). At least one serum protein, LPS-binding protein (LBP), catalyzes the effects of LPS on monocytes that are mediated through the CD14 receptor (16, 18).
We hypothesized that the stimulatory effects of HBP on the LPS-induced release of TNF-α from monocytes (12) are mediated by specific binding of HBP to monocytes and up-regulation of membrane-bound CD14. We demonstrate that HBP alone induces the release of TNF-α and that HBP has an additive effect on LPS-induced release of the proinflammatory mediators TNF-α and PGE₂. HBP has a high affinity to monocytes and is internalized within 30 min. Contrary to our hypothesis, HBP did not increase CD14 expression.

Materials and Methods
Preparation of human recombinant HBP

HBP was expressed in Spodoptera frugiperda (SPF) cells (Invitrogen, Abingdon, U.K.) and purified as previously described (12). Briefly, we constructed a 770-bp BamHI-HindIII fragment from a human bone marrow cDNA library (Clontech Laboratories, Heidelberg, Germany) using PCR technology. We then inserted the fragment into the baculovirus transfer vector pBlueBacIII (Invitrogen), which resulted in the transfer plasmid pSX556. SPF cells were transfected using linear Autographica california nuclear polyhedrosis virus DNA and transfection plasmid (Invitrogen). We collected the insect cell culture medium 3 to 4 days after transfection and purified HBP by glass microfiber filtration (GF/A, Whatman, Maidstone, U.K.), CM-Sepharose cation exchange columns, and Sephadex G-25 gel filtration columns (Pharmacia, Zurich, Switzerland).

Reagents and mAbs

Mouse anti-human CD14, FITC, and phycoerythrin (PE)-coupled Mo2 (Coulter, Hialeah, FL) were used to tag monocytes or measure CD14 expression on monocytes. Purified mouse anti-human CD14 (MY4, 20 μg/ml, Coulter, Hialeah, FL) was used to block CD14. Mouse anti-human CD18 (unlabeled and FITC-coupled clone 1B4, Ancell, Bayport, MN) was used to block CD18 and to assess CD18 expression on monocytes, respectively. Isotype-matched FITC- and PE-coupled Ig (Becton Dickinson, Rutherford, NJ) were used as a control for CD marker expression. Isotype-matched IgG2 (Ancell) was used as a control in the Ab studies. Heparin (sodium heparinate) was obtained from Elkin-Sinn (Cherry Hill, NJ). Esherichia coli 011:B4 LPS, EDTA, dextran-500, RPMI with glutamine, and HBSS with Ca²⁺ and Mg²⁺ were purchased from Sigma Chemical (St. Louis, MO). The Limulus amebocyte lysate assay was purchased from Associates of Cape Cod (Woods Hole, MA). Sterile and endotoxin-free FCS (LPS concentration <0.03 ng/ml) was purchased from BioWhittaker (Walkersville, MD), fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

Affinity studies for LPS and HBP

Whole blood was collected from healthy volunteers and stored in acid citrate dextrose vacutainers at room temperature until the studies were conducted. For the LPS binding studies, we used 10 μg/ml FITC-LPS E. coli 055:B5 (Sigma Chemical). To assess the affinity of HBP to leukocytes, we used a concentration of 10 μg/ml FITC-HBP, a dose based on the published data. Initial affinity and time response studies were determined in 12 mm × 75 mm polypropylene tubes with a final volume of 600 μl/tube in a shaking water bath at 37°C.

In subsequent studies, we determined the affinity of FITC-HBP and FITC-LPS in 50 μl of whole blood in microcentrifugation tubes at 37°C with 5% CO₂ or at 4°C. Meticulous care for temperature control was necessary in the later experiments. In the affinity studies performed at 4°C, the samples were placed on melting ice and incubated in the refrigerator. Pre-incubation was performed for 60 min, and coinoculation consisted of the concurrent addition of the reagents, unless stated otherwise. At the end of the experiments, erythrocytes were removed by hypotonic lysis, samples were washed twice with FITA-azide (Becton Dickinson, Cockeysville, MD), fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

Monocyte isolation and culture

Human monocyte cells were isolated by dextran sedimentation and density gradient centrifugation (19). Briefly, whole blood was collected in EDTA vacutainers, and one part of 6% dextran-500 in 0.9% saline (w/v) 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-Monoocyte (1068 gradient; Accurate Scientific, Westbury, NY). The gradient was centrifuged at 600 × 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% FCS. The cell suspension was centrifuged for 7 min at 600 × g and eventually resuspended in culture medium. Culture medium (RPMI 1640 with glutamine) was supplemented with 1% FCS (BioWhittaker), 1% antibiotics (100 μg/ml streptomycin, 100 μg/ml penicillin; BioWhittaker), and 1% antimycotics (0.25 μg/ml, amphotericin B; BioWhittaker). The cells were counted with a hemocytometer, and the percentage of CD14-positive monocytes was assessed by flow cytometry. A total of 7.2 × 10⁶ cells in 1 ml supplemented culture medium was added to each well (24-well plate from Costar, Cambridge, MA) and incubated at 37°C with 5% CO₂. The LPS concentration in the supplemented culture medium was <0.03 ng/ml, as assessed with the Limulus amebocyte lysate assay.

Flow cytometry

A FACScan emitting an argon laser beam at 488 nm (Becton Dickinson, Immunocytometry Systems, San Jose, CA) was used. Fluorescence values were collected after gating cells based on the combination of forward scatter (FSC) and sideways light scatter (SSC). A total of 5000 cells was analyzed per tube, and acquired data were processed using CellQuest version 1.2 software (Becton Dickinson, Immunocytometry Systems). The fluorescence distributions were displayed as single histograms for fluorescence measured at 530 nm (FL1-H) or fluorescence measured at 580 nm (FL2-H). The percentage of fluorescent cells and the mean fluorescence intensity (MFI) were determined in each case. The signals were acquired in a linear mode for FSC and SSC and in a logarithmic mode for FL1-H and FL2-H. The threshold levels were set according to the negative control. The gates for human monocytes, lymphocytes, and neutrophils were set according to the standard position in the SSC and the FSC (20).

Measurement of proinflammatory mediators

Monocytes were isolated as described, transferred to microcentrifugation tubes, and incubated with HBP (10 μg/ml) for 15 min, 30 min, 45 min, 60 min, and 180 min. Samples were transferred to coverglass chambers (Nunc, Naperville, IL), and fluorescence was assessed with confocal microscopy (Meridian, Okemos, MI). In experiments with dual labeling (60 min incubation with FITC-HBP and labeling of monocyte CD14 with Mo2-PE), the calibration and accuracy of the two filters (530 nm for FITC-fluorescence; 580 nm for PE-fluorescence) was tested using anti-CD14 (Mo2-PE), anti-CD18 (IB4-FITC), and a combination of both mAbs. Data were processed with the software provided by the manufacturer (Meridiani) and assembled with Photoshop 4.0 (Adobe Systems, San Jose, CA).

Statistical analysis

Statistical significance was determined with ANOVA and Fisher’s proba-ble least-squares difference analysis (Statview 4.5, Abacus Concepts, Berkeley, CA) to compare data between multiple groups at each time period. Student’s t tests was used to compare the data between two groups. A p value of <0.05 was considered significant.

Results

Response of human monocytes to LPS and HBP

In initial experiments, we assessed dose responses for LPS and HBP. Isolated human monocytes were incubated for 24 h with increasing concentrations of LPS or HBP. TNF-α release was measured by ELISA. The TNF-α values for each donor are shown in Figure 1 and demonstrate different individual sensitivities to LPS (Fig. 1A) and HBP (Fig. 1B). Donor A (solid circles) first responded to 1.25 ng/ml LPS, whereas the other donors needed a LPS concentration four times higher to release a similar amount of TNF-α (Fig. 1A). Donor A also had one of the highest responses to HBP (Fig. 1B). Donor E had a relatively low response to LPS and a low-to-moderate response to HBP. Overall, LPS produced an increase in TNF-α release that was significant at LPS concentrations of 5 ng/ml and reached a plateau between 10 and 20 ng/ml. Because of the large variability in the individual responses, HBP did not produce a significant mean increase in TNF-α until 10 μg/ml was used. Differences in individual responses to HBP could not be attributed to the sex of the subject (data not shown).
In subsequent experiments, effective doses of HBP (10 μg/ml) were incubated with freshly isolated human monocytes in the absence and presence of LPS (10 ng/ml or 10 μg/ml), and the effluent was analyzed for TNF-α and PGE₂ (Fig. 2). The release of TNF-α (Fig. 2A) was significantly increased after 10 μg/ml HBP (gray bar) and 10 ng/ml or 10 μg/ml LPS (hatched bars). The combination of HBP with either dose of LPS (black bars) significantly increased the release of TNF-α compared with LPS only (hatched bars). The release of PGE₂ was less sensitive to HBP or LPS (Fig. 2B). HBP (gray bar) did not significantly increase PGE₂ production, and only the high concentration of LPS (hatched bars) induced PGE₂ release. However, the combination of HBP with either dose of LPS (black bars) increased PGE₂ above that obtained with LPS alone (hatched bars).

Because HBP has a binding domain for LPS (15), it was possible that the sequence in which HBP and LPS were added to the isolated cells could change the TNF-α production from monocytes. We therefore investigated the impact of the sequential addition of HBP and LPS on TNF-α release in a separate experiment. Five donors were used in each of three groups: 1) 30 min of monocyte incubation with 10 μg/ml of HBP, and then 10 ng/ml of LPS was added for 24 h (TNF-α: 2690 ± 411 pg/ml); 2) 30 min of LPS exposure followed by 24 h of HBP exposure (TNF-α: 2454 ± 318 pg/ml); and 3) a group in which HBP and LPS were preincubated for 30 min before the mixture was added to monocytes for 24 h (TNF-α: 2454 ± 318 pg/ml). The results were not statistically different, suggesting that sequentially adding HBP and LPS to monocytes does not alter the functional effect of HBP on the enhancement of LPS-induced TNF-α production.

**Binding of HBP to leukocytes**

The effects of HBP on the release of TNF-α and PGE₂ from monocytes could result from the binding of HBP to the monocyte. To test this, HBP was conjugated with FITC (FITC-HBP), and the conjugate was incubated with whole blood for 60 min at 37°C. HBP-binding to various leukocyte populations was determined by flow cytometry. Leukocytes were gated into three groups (Fig. 3A) representing lymphocytes (Fig. 3B), monocytes (Fig. 3C), and granulocytes (Fig. 3D). FITC-HBP showed a differential pattern of affinity to the leukocytes. Monocytes had the highest affinity for FITC-HBP, as reflected by a shift in fluorescence intensity units when compared with control FITC-IgG (Fig. 3C). Both lymphocytes and granulocytes showed very little binding of HBP as reflected by the very small shift in fluorescence compared with that of the control FITC-IgG.

The time interval required for FITC-HBP to bind to the leukocyte population is shown in Figure 4. At 2 min, the percentage of monocytes with FITC-HBP binding began to increase. At 10 min, this increase was significantly greater than that of the lymphocytes or granulocytes, and it peaked at 30 min. Lymphocytes and granulocytes showed no significant binding to FITC-HBP, even after 180 min of incubation.

**Characterization of HBP binding to monocytes**

**Role of CD14.** The affinity of HBP to monocytes could be mediated by binding to the monocyte differentiation marker and LPS receptor CD14, because HBP is known to bind to the lipid A moiety of LPS (14, 15). However, preincubation of whole blood with the anti-CD14 mAb (MY4, 20 μg/ml) did not alter FITC-HBP binding to monocytes over a period of 180 min (Fig. 5, A and B).

**Role of CD18.** CD18 is also a receptor that binds LPS (21). Blocking of CD18 with a anti-CD18 mAb (IB4, 15 μg/ml) did not alter FITC-HBP fluorescence from monocytes. MFI for anti-CD18-treated monocytes (n = 5 donors) was 154 ± 26, and MFI for control IgG-treated monocytes (n = 5 donors) was 144 ± 16.

**Effect of LPS.** LPS (10 μg/ml) significantly reduced early FITC-HBP affinity to monocytes (after 1 to 15 min) but not at later time points (30 and 60 min) (Fig. 6A, solid squares).

**Effect of heparin.** HBP has a high affinity for heparin (10, 15). It was therefore possible that heparin would alter the binding characteristics of HBP to monocytes. Coincubation of heparin (1 mg/ml) with FITC-HBP completely blocked FITC-HBP binding to monocytes (p < .0001, Fig. 6, A and B).
Effect of temperature. The binding of HBP to monocytes could be mediated by mechanisms requiring energy for receptor presentation or HBP internalization. Indeed, our results (Fig. 6C) demonstrated that FITC-HBP had no affinity to monocytes at 4°C; only 3 ± 1% of monocytes (n = 5 donors) were fluorescent (Fig. 7), with a fluorescence intensity of 21 ± 1, a value that corresponds to cellular autofluorescence. However, at 37°C, 89 ± 6% monocytes (n = 5 donors) were fluorescent (Fig. 7), with a fluorescence intensity of 144 ± 16 (p < .0001, mean ± SEM). In contrast, changes in temperature from 37°C to 4°C did not change the binding of Mo2 (anti-CD14 mAb) or FITC-LPS to monocytes (Fig. 7). These results demonstrated completely different binding properties of LPS and HBP to human monocytes.

Localization of HBP in monocytes

In separate experiments, monocytes were isolated using 1-Step-Monocyte, a method that yields a 90% pure monocyte population. Confocal microscopy was used to localize fluorescence from FITC-HBP in monocytes. Experiments performed over a period of 15 to 240 min demonstrated fluorescence on the cell surface at 15 min and within the monocytes by 30 min (Fig. 8A). To show that HBP was localized within CD14-positive monocytes, we incubated monocytes for 60 min with FITC-HBP and labeled CD14 on the monocyte membrane with Mo2-PE (25 min at 4°C). The use of two filters during confocal microscopy (530 nm for FITC and 580 nm for PE) allowed us to assess both FITC and PE fluorescence within the same monocyte. The results confirmed the findings of the initial studies and demonstrated FITC-fluorescence inside a ring of red-labeled monocyte membranes (Fig. 8B).

Modulation of monocyte CD14 or CD18 expression by HBP and LPS

Because HBP binding was not altered by blockade of CD14 or CD18, HBP could have an effect on CD14 or CD18 expression as a mechanism to increase monocyte production of inflammatory mediators. In these experiments, HBP was incubated with monocytes at three different concentrations (0.1, 1, and 10 μg/ml). HBP did not change CD14 (Fig. 9A) or CD18 (Fig. 9B) expression at any of the concentrations used. However, the expression of CD14 was altered by LPS (Fig. 10). After 4 h of incubation with 10 μg/ml of LPS, there was a small but significant decrease in the number of cells expressing CD14 (Fig. 10A). The number of cells expressing CD14 then continued to decrease over the 24-h experiment. There was also an increase in the MFI at 1 and 4 h after incubation with 10 μg/ml LPS (Fig. 10B), indicating an increased expression of CD14 per cell. Longer incubation times (18 and 24 h), however, resulted in a decreased MFI or a reduction in the number of CD14 expressed per cell. In a separate experiment, we demonstrated that HBP did not further modulate the LPS-induced
changes in monocyte CD14 expression after 4 and 24 h (n = 5 donors; data not shown).

Discussion

We have established that HBP, a protein released from activated neutrophils, activates human monocytes to release TNF-α and that HBP increases the LPS-induced release of TNF-α and PGE₂ (Fig. 2). Our results demonstrate that HBP, when compared with lymphocytes or granulocytes, has a high affinity to CD14-positive monocytes (Figs. 3 and 4) but not to the LPS receptor CD14 (Fig. 5). Furthermore, we demonstrate that HBP is internalized in monocytes (Fig. 8), and that HBP increases the production of proinflammatory mediators by mechanisms other than increased monocyte CD14 or CD18 expression (Fig. 9).

We found that HBP induced TNF-α release in the absence of LPS. These findings differ from the results reported by Rasmussen et al. (12). Those authors report that HBP has a potentiating effect on LPS-induced cytokine production. They found that 20 µg/ml of HBP and, surprisingly, 10 ng of LPS alone did not increase the release of IL-1, IL-6, or TNF-α from isolated monocytes (12), whereas we found large TNF-α responses at lower doses of HBP and the same dose of LPS. Interestingly, three donors in our experiment (Fig. 1) were very low responders to HBP and low responders to LPS. Rasmussen et al. (12) might have been looking at a single low responding individual. The variability in the current experiments in HBP responsiveness (Fig. 1) could be similar to the variability for LPS-induced TNF-α production, which depends on genetic factors (22, 23). While the data suggest that the sensitivity to HBP-induced TNF-α production is also genetically determined, we found no evidence of a sex-linked sensitivity. There was also

FIGURE 6. Inhibition of HBP affinity to monocytes. LPS, heparin, and low temperature (4°C) reduced FITC-HBP binding to human monocytes in whole blood. A, Demonstrates FITC-HBP (10 µg/ml) fluorescence on human monocytes in the presence of 0.9% saline, the initial reduction of FITC-HBP affinity in the presence of 10 µg/ml of LPS, and the abrogation of FITC-HBP affinity to human monocytes in the presence of 1 mg/ml of heparin, or when experiments were performed at 4°C. Values are mean ± SEM (n = 5 donors). *, p < 0.05 determined with t test by comparing the LPS, heparin, and 4°C groups to the saline control group. Representative histograms are shown for heparin-treated monocytes (B) and experiments performed at 4°C (C). Control groups are shown in white and test groups in gray.

FIGURE 7. Effect of temperature on HBP, Mo2, and LPS affinity to monocytes. Whole blood was incubated with FITC-HBP (10 µg/ml), anti-CD14 mAb Mo2-PE (5 µg/ml), or FITC-LPS (10 µg/ml) for 60 min at two different temperatures: 4°C (hatched bars) and 37°C (solid bars). Binding to monocytes was analyzed by flow cytometry. Values of percentage of monocytes with fluorescence are shown as mean ± SEM (n = 5 donors). *, p < 0.05 determined by paired t test.

FIGURE 8. Localization of FITC-HBP fluorescence in monocytes. Monocytes were isolated by density gradient centrifugation, exposed to 10 µg/ml of FITC-HBP at 37°C, and fixed in 1% paraformaldehyde, and confocal microscopy was performed. A series of horizontal “slices” of monocytes are shown. The light microscopic view is displayed in the upper right corners of each panel. Subsequent views start from the top of the cell and show green FITC-HBP fluorescence on the outside as well inside the monocyte. The lower right photomicrograph in each panel displays the bottom of the cell. A, A monocyte 30 min after FITC-HBP incubation. B, Monocytes incubated with FITC-HBP for 60 min and subsequently stained with a red fluorescent monocyte-specific Ab (anti-CD14, Mo2-PE). The red ring represents the cell membrane, and the green-yellow fluorescence from inside the cell demonstrates internalization of HBP.

FIGURE 9. Effect of HBP on monocyte CD14 and CD18 expression. HBP was added to whole blood in three different doses: 0.1, 1, and 10 µg/ml. Monocytes were analyzed for CD14 and CD18 expression over a 24-h period by flow cytometry. The expression of monocyte CD14 (measured with FITC-conjugated anti-CD14 mAb Mo2 (A)) and monocyte CD18 (measured with FITC-conjugated anti-CD18 mAb IB4 (B)) was not altered by HBP and is displayed as the difference of MFIs compared with 0.9% saline control. Values are mean ± SEM (n = 3 donors).
the possibility that the variability in our data and/or the positive response to HBP alone could be due to LPS contamination. We measured the LPS concentration in the HBP stock solution and calculated a final contamination of 12 to 24 pg/ml LPS in the culture medium. However, our LPS dose-response study (Fig. 1A) demonstrated that a 100-fold higher concentration of LPS (1.25 ng/ml) did not by itself produce a significant increase in TNF-α production. This indicates that HBP, and not LPS contamination, dramatically increased the TNF-α production that was induced by 10 ng/ml of LPS (Fig. 2).

LPS activates monocytes via membrane-bound CD14 on monocytes (16, 24), an effect that is increased in the presence of the serum protein LBP (18). The functional LPS receptor appears to be a multimeric receptor that consists of the glycosylphosphatidylinositol-anchored CD14 and a presently unidentified transmembrane protein (16). Interestingly, substances other than LPS, such as uronic acid polymers (25) or other bacterial cell wall products (such as soluble peptidoglycans (26) or lipooraminomannan from Mycobacterium tuberculosis (27)), induce cytokine production in monocytes via CD14.

These multimeric properties of the LPS receptor suggest that HBP could act through CD14 to activate monocytes and release TNF-α and PGE2 (Fig. 2). This idea was strengthened by the fact that HBP had a high affinity to monocytes (Figs. 3 and 4). Therefore, we investigated whether HBP binds to CD14 on monocytes. Inhibition of the interaction of LPS-LBP with CD14 can be achieved with a number of mAbs, including the broadly reactive MY4 (28). We used 20 µg/ml of MY4 to block CD14, a concentration that completely inhibited FITC-LPS binding to CD14 (29). However, MY4 did not induce a reduction of HBP binding to monocytes (Fig. 5). Therefore, we conclude that HBP does not bind to the CD14 epitope recognized by MY4. This is further supported by the fact that both LPS and anti-CD14 mAbs bind to constitutively expressed CD14 on monocytes at 4°C, whereas HBP does not bind to monocytes at 4°C (Fig. 7).

Others have shown that the number of membrane-bound CD14 correlates with the production of LPS-induced cytokines such as IL-8 (30). We investigated the modulation of monocyte CD14 expression by HBP and LPS (Figs. 9 and 10). Our results showed that HBP did not modulate monocyte CD14 expression, suggesting that up-regulation of CD14 is not the mechanism responsible for the effect of HBP on the enhancement of LPS-induced TNF-α release. However, LPS induced a biphasic pattern of monocyte CD14 expression (Fig. 10), with up-regulation of CD14 at 4 h and down-regulation of CD14 at 18 and 24 h. The early up-regulation of monocyte CD14 was also reported by Marchant et al. (31), who postulated a translocation of CD14 from an intracellular pool to the cell surface. A down-regulation of CD14 at 18 h was described by Wright (32) and by Landmann et al. (33). However, a 2-day incubation of LPS caused increased levels of CD14 mRNA, membrane-bound CD14, and soluble CD14 (33). The authors concluded that CD14 is transcriptionally up-regulated by LPS (33). Studies by Bazil and Strominger (34) showed that LPS down-regulates monocyte CD14 expression by shedding the receptor from the surface. In our experiments, HBP binding to monocytes was not inhibited with CD14 blockade (Fig. 5), and HBP did not alter monocyte CD14 expression when compared with the control group (Fig. 9). In addition, in the current experiments, the binding characteristics of LPS and HBP are radically different, since binding of FITC-HBP to monocytes was completely inhibited at 4°C, but binding of FITC-LPS was not (Fig. 7). Therefore, it does not appear that the effect of HBP can be attributed to an effect on the LPS receptors CD14 or CD18.

Initially, binding of HBP to monocytes was reduced in the presence of LPS, but there was no significant difference after 30 min (Fig. 6A). We therefore tested the impact of different sequential additions of LPS and HBP and found no differences in TNF-α production after 24 h. This indicates that the initial binding of HBP and LPS to monocytes does not alter the functional effect of HBP on the enhancement of LPS-induced TNF-α production. We propose that HBP, inside the cell (Fig. 8), activates intracellular pathways, with the potential to increase the LPS-induced signaling cascade derived from CD14. This hypothesis of intracellular influence on signaling by HBP is supported by the recent study of Pereira (35), who demonstrated that HBP regulates vascular endothelial cell protein kinase C in both a time- and dose-dependent manner.

Campbell (36) used two radiolabeled serine proteases with homology to HBP, leukocyte elastase, and cathepsin G, and also radiolabeled lactoferrin (another glycoprotein released from neutrophil granules), to study binding to alveolar macrophages. Saturable binding of all three proteins at 0°C was described, and the three proteins bound to a similar number (54–73 × 106) of sites per cell. Campbell argued that these receptors would be ideally suited to clear neutrophil granule contents from the extracellular space in inflamed tissues (36). Our affinity studies of HBP to human monocytes demonstrate a lack of binding at 4°C (Fig. 7) and argue for an energy-requiring step such as receptor turnover or HBP internalization. In contrast to Campbell’s “clearance” hypothesis, we showed that the binding and internalization of HBP to monocytes was concurrent with an increased TNF-α and PGE2 production and appeared to be, at least, additive to the effects of LPS on the production of these two mediators. These findings suggest that the binding and internalization of free HBP is not only a clearance phenomenon, but may represent an inducer and possibly an amplifier phenomenon of monocyte activation.

CD18 is also a possible LPS receptor (17) that binds particulate LPS but is unnecessary for the responses of macrophages to LPS (24). Blocking of CD18 with the anti-CD18 Ab IB4 did not inhibit...
HBP binding to monocytes in our experiments (Fig. 9). Wright et al. (24) demonstrated that blocking CD18 did not reduce LPS-induced TNF-α synthesis. These findings indicate that CD18 is not an important receptor in the HBP-mediated increase of monocyte-derived inflammatory mediators and is not a receptor for HBP.

To date, the mechanism by which HBP induces the release of TNF-α and PGE₂ and increases the LPS-induced release of these two proinflammatory mediators in monocytes remains unknown. However, we have demonstrated that the mechanism does not include binding to CD14 or modulation of CD14 expression on monocytes, which is contrary to our original hypothesis. The lack of HBP binding to monocytes at 4°C argues for an energy-dependent step in this process that may consist of internalization of HBP.

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