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Lipopolysaccharide Binding Protein and Serum Amyloid A Secretion by Human Intestinal Epithelial Cells During the Acute Phase Response[1]

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The acute phase proteins LPS binding protein (LBP) and serum amyloid A (SAA) are produced by the liver and are present in the circulation. Both proteins have been shown to participate in the immune response to endotoxins. The intestinal mucosa forms a large surface that is continuously exposed to these microbial products. By secretion of antimicrobial and immunomodulating agents, the intestinal epithelium contributes to the defense against bacteria and their products. The aim of this study was to explore the influence of the inflammatory mediators TNF-α, IL-6, and IL-1β on the release of LBP and SAA by intestinal epithelial cells (IEC). In addition, the induction of LBP and SAA release by cell lines of intestinal epithelial cells and hepatic cells was compared. The data obtained show that in addition to liver cells, IEC also expressed LBP mRNA and released bioactive LBP and SAA upon stimulation. Regulation of LBP and SAA release by IEC and hepatocytes was typical for class 1 acute phase proteins, although differences in regulation between the cell types were observed. Endotoxin did not induce LBP and SAA release. Glucocorticoids were demonstrated to strongly enhance the cytokine-induced release of LBP and SAA by IEC, corresponding to hepatocytes. The data from this study, which imply that human IEC can produce LBP and SAA, suggest a role for these proteins in the local defense mechanism of the gut to endotoxin. Furthermore, the results demonstrate that tissues other than the liver are involved in the acute phase response. ***The Journal of Immunology,*** **1999, 163: 2792–2798.**

Lipopolysaccharide binding protein (LBP)[3] is an acute phase protein that strongly modulates the response to endotoxins, which are present in the outer membrane of Gram-negative bacteria. LBP enhances the recognition of endotoxin and bacterial surfaces by the immune system (1). Other studies demonstrated that an important function of LBP is to mediate the neutralization of endotoxin by lipoproteins (2, 3). Furthermore, it was demonstrated that high concentrations of LBP decrease endotoxin activity and protect against septic shock caused by Gram-negative bacteria (4).

The intestinal lumen contains high amounts of endotoxin, and the intestinal mucosa forms the interface between this potentially harmful material and the interior of the host. The intestinal epithelium contributes to the immunologic defense by secretion of proinflammatory cytokines (5) and microbicidal agents such as defensins (6). Although the liver has been held responsible for the production of circulating acute phase proteins, there is recent evidence for the synthesis of acute phase proteins in the intestinal mucosa as well (7, 8). This prompted us to study whether the release of LBP and the major acute phase protein serum amyloid A (SAA), which is involved in the routing and binding of high density lipoprotein (HDL) to inflammatory cells (9), is part of the epithelial response to inflammation. In the current study we examined the regulation of LBP and SAA secretion by a number of intestinal epithelial cell lines in detail and compared it to the regulation in hepatocytes. The data obtained show that intestinal epithelial cells can release both LBP and SAA, which strongly implicates a role for these proteins in the local inflammatory processes in the gut.

Materials and Methods

Reagents and Abs

Human recombinant TNF-α was provided by BASF/Knoll (Ludwigshafen, Germany); human recombinant IL-6 was provided by Prof. W. Sebald (Physiologisch-Chemisches Institut de Universität Wurzburg, Wurzburg, Germany); human rIL-1β was a gift from Dr. S. Gillis (Immunex, Seattle, WA); dexamethasone was a gift from Merck Sharp, & Dohme (Haarlem, The Netherlands); MTT and LPS (from *Escherichia coli*, serotype 055:B5) were purchased from Sigma (St. Louis, MO). Human recombinant LBP was produced by transfected CHO cells, provided by Dr. P. Tobias (Research Institute of Scripps Clinic, La Jolla, CA). Polyclonal Abs to human rLBP were obtained by immunizing rabbits with human rLBP. After protein A purification, IgG was biotinylated. A specific anti-human LBP mAb HM14 was obtained by immunizing mice with human rLBP following classical procedures. The selection and properties of the mAb will be discussed in detail elsewhere. In short, this mAb was selected on basis of its reactivity with both free human LBP and human LBP that has formed a complex with LPS. The SAA immunoassay was provided by Dr. P. C. Limburg (Department of Rheumatology, University Groningen, Groningen, The Netherlands).

Culture and stimulation of cells

The cell lines Caco-2, Colo-205, and T-84 were obtained from American Type Culture Collection (Manassas, VA; ATCC HTB-37, CCL-222, and CCL-248 respectively). Human colonic adenocarcinoma Caco-2 cells undergo in vitro differentiation and express structural characteristics and functional properties typical of small intestinal enterocytes. The Colo-205 and T-84 cell lines are human colonic carcinoma cell lines. The small

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[3] Abbreviations used in this paper: LBP, LPS binding protein; IEC, intestinal epithelial cells; SAA, serum amyloid A; HDL, high density lipoprotein; TMB, tetramethylbenzidine.

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intestinal epithelial cell line Int-407 was obtained from the European Collection of Cell Cultures (Wiltshire, U.K.; ECACC 85051004). Human hepatoma (HuH-7) cells were a gift of Dr. K. C. H. Fearon, Department of Surgery, University of Edinburgh (Edinburgh, U.K.). Cells were cultured at 37°C with 5% CO_2. HuH-7 and Colo-205 cells were cultured in RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 10% FCS (HyClone, Logan, UT). Caco-2 cells were cultured in DMEM (Life Technologies) supplemented with 10% FCS (HyClone), 1.0 mM sodium pyruvate (Life Technologies), and 0.1 mM nonessential amino acids (Life Technologies). Int-407 cells were cultured in MEM with Earle's salts (Life Technologies) supplemented with 10% FCS (HyClone) and 0.1 mM nonessential amino acids (Life Technologies). All culture media were supplemented with 100 IU of penicillin/ml and 100 μg of streptomycin/ml (Life Technologies). Cell lines were maintained in 25-cm^2 flasks (Costar, Cambridge, MA). Cultures were split when they reached 80% confluence. For experiments designed to investigate the effect of cytokines on the release of LBP and SAA, cells were plated at a density of 1×10^4 cells/well on 96-well flat-bottom tissue culture plates (Costar). Cells were cultured in complete medium as described above. The medium was changed 48 h after plating and then every other day. After 2 wk, Caco-2 monolayers display morphologic and functional properties similar to those found in normal small intestinal enterocytes (10). Therefore, all experiments with Caco-2 cells were conducted between 14–17 days after seeding of the cells. Experiments with Colo-205, T-84, Int-407, and HuH-7 cells were conducted after confluence was established microscopically. At the start of each experiment, culture medium was removed, and complete medium, supplemented with IL-1β, IL-6, TNF-α, LPS, or dexamethasone, as single agents or in combination as given in *Results*, was added. Stimulation with LPS was performed in the presence of human serum. At the end of the incubation period, the medium was removed and centrifuged to remove cells and cell debris. Cell number and viability were determined using the MTT method as described previously (11). To determine whether the presence of serum affected the cytokine-induced LBP and SAA release, Caco-2 cells were stimulated under serum-free conditions using a protocol similar to stimulations conducted in the presence of serum.

For experiments designed to determine whether endothelial cells also posses the capacity to release LBP and SAA, HUVEC were isolated from fresh umbilical cords by treatment with collagenase type I (Sigma). Cells were seeded into fibronectin-coated tissue culture flasks. For stimulation experiments cells were seeded in 96-well plates at a density of 1×10^4 cells/well and were cultured for 2–3 days to confluence. Cells were stimulated in a protocol similar to IEC and hepatocytes.

Detection of LBP mRNA

Total cellular RNA was isolated from quiescent and stimulated Caco-2 and HuH-7 cells using a Quick Prep Total RNA Extraction Kit (Pharmacia, Uppsala, Sweden). cDNA was obtained by RT of total RNA. PCR of β_2m was employed for standardization of the different RNA samples as described by Kloppenburg et al. (12). Human LBP-targeted PCR was performed in a 25-μl reaction mixture containing 100 μM of each dNTP, 200 nM of primers, and 0.5 U of Taq DNA polymerase (Perkin-Elmer/Cetus, Emeryville, CA). The primers were described by Su et al. (7): sense primer, 5′-AGG-GCC-TGA-GTC-TCA-GCA-TCT-3′; and antisense primer, 5′-CAG-GCT-GGC-CGT-GTT-GAA-GAC-3′. Thirty-five cycles were run under the following conditions: 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min. The reaction product was analyzed on a 1.2% agarose gel containing ethidium bromide. A mock PCR (without cDNA) was included to exclude contamination.

Immunoassays

LBP and SAA release was evaluated using sandwich ELISAs. LBP was determined as described previously (13). In short, plates (Nunc-Immuno Plate Maxisorp, Roskilde, Denmark) were coated with polyclonal anti-human LBP Abs. Supernatants of the cells and a standard dilution series of rLBP were added to the plate. Detection was performed with a biotinylated polyclonal rabbit anti-human LBP IgG, followed by peroxidase-conjugated streptavidin (Zymed, San Francisco, CA) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The detection limit was 200 pg/ml.

SAA was quantified as described previously (14). In short, SAA-specific mAb Reu 86.5 was used as capture reagent. The amount of SAA bound to the wells was quantified by incubation with a HRP-labeled monoclonal anti-SAA Ab (Reu 86.1) followed by TMB substrate. The detection limit for the SAA assay was 100 pg/ml.

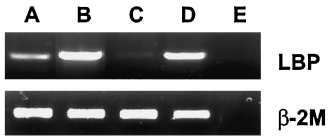

FIGURE 1. Human LBP-targeted RT-PCR amplification of RNA from Caco-2 and HuH-7 cells. Cells were stimulated for 24 h with a mix of IL-1β (5 ng/ml), IL-6 (100 ng/ml), and TNF-α (100 ng/ml). Controls consisted of unstimulated cells. PCR for β_2m (β-2 M**)** was employed for standardization of the samples. *Lane A*, Unstimulated HuH-7 cells; *lane B*, HuH-7 cells after stimulation; *lane C*, unstimulated Caco-2 cells; *lane D*, Caco-2 cells after stimulation; *lane E*, blank.

Characterization of LBP released by Caco-2 cells

To confirm the identity of LBP released by Caco-2 cells, the protein was isolated from the medium by selective affinity immunosorption, followed by determination of the m_r by Western immunoblotting. For this purpose the newly developed anti-LBP mAb HM14 was cross-linked to NHS-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Conditioned culture medium of Caco-2 cells was applied to the anti-LBP column, and the unbound proteins were washed out with PBS. Glycine-HCl buffer (pH 2.5) was used to elute bound LBP. For comparison, rLBP was isolated from the culture medium of transfected CHO cells by the same method. Gel electrophoresis of Caco-2-derived LBP, rLBP, and a prestained low m_r marker was performed using 10–15% gradient polyacrylamide gels (Pharmacia) run in a Pharmacia Phast system followed by electrophoretic transfer onto an Immobilon-P membrane (Millipore, Bedford, MA). LBP bands were detected using a biotin-labeled polyclonal Ab to human LBP followed by peroxidase-labeled streptavidin and 3,3′-diaminobenzidine substrate.

The bioactivity of LBP produced by Caco-2 cells was determined by measuring the LPS binding capacity. For this purpose Immuno-Maxisorp plates were coated with the monoclonal anti-human LBP Ab HM14. Next, dilution series of LBP produced by Caco-2 cells and rLBP were added, followed by biotinylated LPS. For biotinylation of LPS a hydrazide-biotin reagent (Pierce, Rockford, IL) was used. Detection occurred with peroxidase-conjugated streptavidin and TMB substrate. The ODs obtained represent the LPS binding capacity of the LBP.

Statistical analysis

Data were analyzed using SPSS software (SPSS, Chicago, IL). All values in the figures and text are expressed as the mean ± SD of *n* observations. Groups were compared by Student's *t* test. Results of experiments designed to study the effects of cytokines and dexamethasone on the secretion of LBP and SAA by Caco-2 and HuH-7 cells were hierarchically analyzed by four-way ANOVA, using dummy regression analysis after square root transformation of data. A *p* value of 0.05 or less was considered statistically significant.

Results

Cytokines induce the synthesis of LBP and SAA by Caco-2 cells

First we investigated whether IEC express LBP mRNA. In vitro differentiated human colonic adenocarcinoma (Caco-2) cells were used as a model of normal human intestinal epithelial cells. The monolayers were stimulated with a combination of IL-1β (5 ng/ml), IL-6 (100 ng/ml), and TNF-α (100 ng/ml). For comparison, human hepatoma (HuH-7) cells were treated in a protocol similar to that used for Caco-2 cells. LBP mRNA was expressed in quiescent Caco-2 and HuH-7 cells in minor quantities. Subsequently, after culture of cells in the presence of cytokines LBP mRNA expression was up-regulated substantially (Fig. 1). The PCR product was of predicted size (565 bp). These data are the first to demonstrate that intestinal epithelial cells express LBP mRNA, which strongly suggests synthesis of LBP by these cells.

Next, the induction pattern of LBP release by Caco-2 cells was studied. After incubation of the cells with cytokines, supernatants were harvested and analyzed. In the absence of cytokines, low amounts of LBP were detectable in the supernatants. A significant

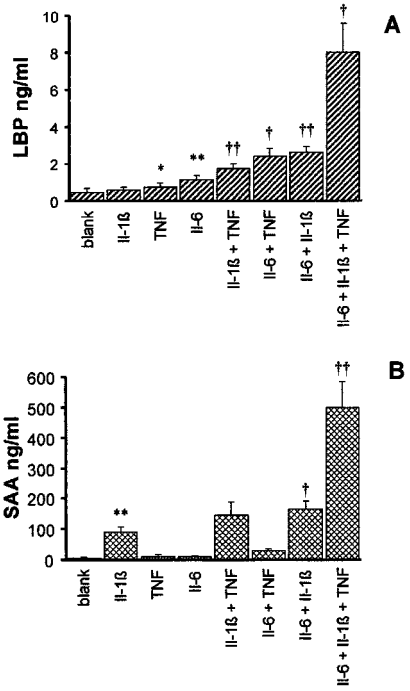

FIGURE 2. Cytokines induce LBP (*A*) and SAA (*B*) release by Caco-2 cells. Cells were incubated with IL-1β (5 ng/ml), IL-6 (100 ng/ml), TNF-α (100 ng/ml), or combinations of these agents as indicated. Controls consisted of unstimulated cells. After 72-h incubation, supernatants were collected, and LBP and SAA release were determined by ELISA. The data of a representative experiment are shown and expressed as the mean ± SD ($n = 6$). ∗, Significant effect of individual cytokines ($p < 0.01$); ∗∗, significant effect of individual cytokines ($p < 0.001$); †, significant positive interaction between cytokines ($p < 0.01$); ††, significant positive interaction between cytokines ($p < 0.001$).

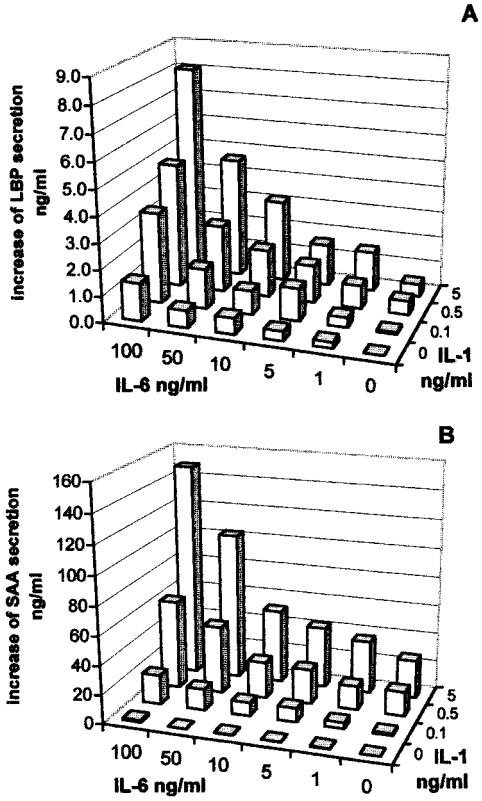

FIGURE 3. Cytokine-induced enhancement of LBP and SAA release by Caco-2 cells is concentration dependent. Caco-2 cells were incubated with increasing concentrations of IL-1β (0–5 ng/ml), IL-6 (0–100 ng/ml), or a combination of both. Controls consisted of unstimulated cells. Supernatants were collected after 72 h of incubation, and LBP and SAA release was determined by ELISA. The data from a representative experiment are shown and expressed as the increase in LBP released (nanograms per milliliter) compared with controls.

($p < 0.001$) up-regulation of LBP release was induced by IL-6 (Fig. 2*A*). IL-1β and TNF-α alone did not induce a marked up-regulation of the LBP release, although both cytokines strongly enhanced the effect of IL-6 ($p < 0.01$) and each other ($p < 0.001$) on LBP release. The strongest up-regulation was induced by the combination of IL-1β, IL-6, and TNF-α ($p < 0.01$; Fig. 2*A*).

The capability of Caco-2 cells to express SAA mRNA was demonstrated by others (15). We investigated the induction pattern of SAA release by Caco-2 cells (Fig. 2*B*). The SAA release of the cells was markedly up-regulated by IL-1β ($p < 0.001$), while treatment with either IL-6 or TNF-α had no significant effect (Fig. 2*B*). However, IL-6 ($p < 0.01$) enhanced IL-1β-induced SAA synthesis, while TNF further up-regulated the effect of the combination of IL-1β and IL-6 ($p < 0.001$). The necessity of IL-1β for apparent induction of SAA release by Caco-2 cells was evident and is typical for class 1 acute phase proteins. In conclusion, Caco-2 cells have the capability to release both LBP and SAA in response to cytokines, while each protein has its own specific induction pattern.

To investigate whether the secretion of LBP and SAA is a general property of in vitro cultured cells, HUVEC were stimulated with proinflammatory cytokines. LBP and SAA was not detected in the supernatants (data not shown), which implicates that LBP and SAA secretion is not a common feature of cells in general or epithelial cells in particular.

Next, the concentration dependency of IL-6 and IL-1β on the induction of LBP and SAA release was studied (Fig. 3). A clear up-regulation of LBP release is mediated in Caco-2 cells by 100 ng/ml Il-6. However, when cells were treated with IL-6 plus IL-1β simultaneously, the enhancement of LBP release was evident at concentrations as low as 1 ng/ml IL-6 (Fig. 3*A*). The data demonstrate that 0.5 ng/ml IL-1β is enough to enhance SAA release. The synergistic effect of IL-6 on IL-1β-mediated SAA release was clear at 5 ng/ml (Fig. 3*B*). Furthermore, synthesis of both proteins increased when concentrations of IL-1β and IL-6 were raised. The data from this experiment indicate that cytokine concentrations that has been previously reported in actively inflamed intestinal mucosa (16, 17) as well as in the mesenteric blood (18) induce LBP and SAA secretion by IEC.

The data obtained in the stimulation experiments were not caused by an effect of cytokines on cell numbers as determined by the MTT method (data not shown). In addition, in the absence of serum, identical induction patterns of LBP and SAA release were observed, although total amounts of LBP and SAA released were lower in serum-deprived conditions (data not shown).

Time kinetics of LBP and SAA release by Caco-2 cells

Fig. 4 shows the time kinetics of LBP and SAA release. After an incubation period of 24 h LBP and SAA concentrations in the supernatants were modest. A substantial increase was seen after 48 h, and at 72 and 96 h LBP and SAA release was further increased. These data are in agreement with the kinetics of LBP and SAA concentrations in the circulation during an acute phase response in man (19, 20).

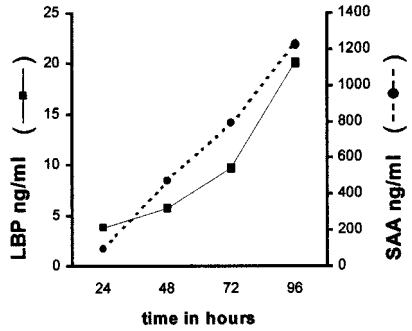

FIGURE 4. Time course of LBP and SAA release by Caco-2 cells after treatment with a combination of IL-1β (5 ng/ml), IL-6 (100 ng/ml), TNF-α (100 ng/ml), and dexamethasone (1 μM). Supernatants ($n = 6$) were collected after 24-, 48-, 72-, and 96-h incubation, and LBP and SAA release was determined by ELISA.

LBP and SAA release by Caco-2 cells is not induced by endotoxin

The gastrointestinal tract contains high amounts of endotoxins that are important inducers of the acute phase response in vivo. To determine whether endotoxins can directly induce the release of LBP and SAA by IEC, endotoxin derived from *Escherichia coli* was added to Caco-2 cells cultured in the presence of human serum containing human LBP and soluble CD14, both necessary for nonimmune cells to respond to endotoxins. Concentrations up to 10 μg/ml endotoxin did not lead to enhanced release of LBP or SAA by Caco-2 cells (data not shown).

To elucidate whether endotoxins are able to enhance the cytokine-induced release of LBP and SAA, Caco-2 cells were incubated with a combination of different concentrations of endotoxin, IL-1β, and IL-6. For all concentrations tested endotoxin did not affect the LBP and SAA release induced by IL-6 or IL-1β (data not shown). In conclusion, these data indicate that LBP and SAA release by IEC is not influenced by endotoxins.

Regulation of LBP and SAA release in Caco-2 cells is different in HuH-7 cells

Acute phase proteins, such as LBP and SAA, are thought to be predominantly liver derived. Therefore, the induction of LBP and SAA release in liver cells was investigated for comparison. To this end, HuH-7 cells were stimulated in a protocol similar to that used for Caco-2 cells (Fig. 5). These experiments revealed that with HuH-7 cells, as with Caco-2 cells, LBP release is induced by IL-6 ($p < 0.001$), whereas SAA release is induced by IL-1β ($p < 0.001$). Despite this conformity, several differences in the induction pattern of both proteins were notable between both cell types. The data demonstrate that IL-1β (5 ng/ml) significantly ($p < 0.001$) up-regulates the LBP release by HuH-7 cells, whereas no effect on LBP release was observed in Caco-2 cells. Furthermore, TNF-α potentiated the LBP and SAA synthesis induced by the combination of IL-6 and IL-1β in Caco-2 cells ($p < 0.01$), whereas a down-regulation of this induction was observed in HuH-7 cells ($p < 0.01$; Fig. 5). In addition, the amount of SAA released by Caco-2 cells exceeded the LBP released by these cells, whereas the opposite was found in HuH-7 cells. As in Caco-2 cells, endotoxin did not evoke significant LBP and SAA release by HuH-7 cells (data not shown).

Dexamethasone enhances cytokine-induced LBP and SAA release by Caco-2 cells

An important feature of the systemic acute phase response is the involvement of glucocorticoids. These glucocorticoids enhance the

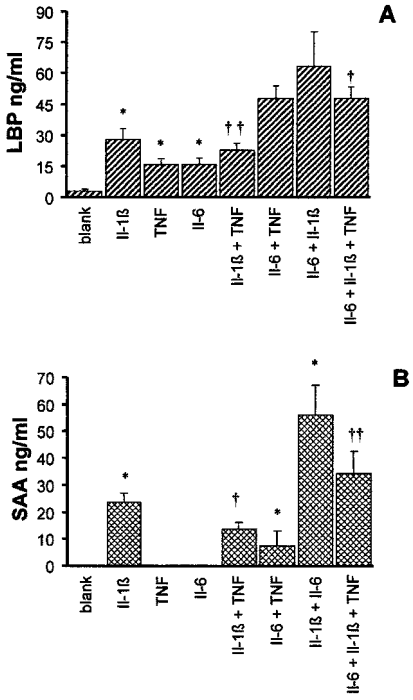

FIGURE 5. Cytokines induce LBP (*A*) and SAA (*B*) release by HuH-7 cells. Cells were incubated with IL-1β (5 ng/ml), IL-6 (100 ng/ml), TNF-α (100 ng/ml), or combinations of these agents as indicated. Controls consisted of unstimulated cells. After 72-h incubation, supernatants were collected, and LBP and SAA release was determined by ELISA. The data from a representative experiment are shown and expressed as the mean $\pm$ SD ($n = 6$). $*$, Significant effect of individual cytokines ($p < 0.001$); †, significant negative interaction between cytokines ($p < 0.01$); ††, significant negative interaction between cytokines ($p < 0.001$).

IL-1-, TNF-, and IL-6-mediated induction of the acute phase response in the liver. To elucidate the effect of glucocorticoids on cytokine-mediated release of LBP and SAA by IEC, Caco-2 cells were stimulated in the presence of 1 μM dexamethasone, a synthetic glucocorticoid. Dexamethasone enhanced the LBP synthesis induced by IL-6 ($p < 0.05$) and markedly enhanced the induction by the combination of IL-6, IL-1β, and TNF-α ($p < 0.01$; Fig. 6*A*). Furthermore, spontaneous SAA release and SAA release induced by the combination of IL-1β and IL-6 were strongly enhanced by dexamethasone (Fig. 6*B*). The enhancement of LBP and SAA release by glucocorticoids was concentration dependent (data not shown). A negative interaction between dexamethasone and the combination of IL-1β, IL-6, and TNF-α on SAA release was found ($p < 0.001$). These data indicate a role for glucocorticoids in the local acute phase response by regulation of the cytokine-mediated LBP and SAA release in the gut.

Characterization of LBP released by Caco-2 cells

To characterize LBP produced by Caco-2 cells the M_r of the isolated protein was determined by Western immunoblotting and compared with that of rLBP. LBP produced by Caco-2 cells migrated as a single band with a M_r of approximately 60 kDa, identical with that of rLBP (Fig. 7).

A characteristic of functional LBP is its capacity to bind LPS. Therefore, the capacity of Caco-2-derived LBP to bind LPS was established. The LBP produced by Caco-2 cells showed a dose-dependent binding of LPS similar to that of rLBP (Fig. 8). These results ascertain the functional capacity of LBP produced by intestinal epithelial cells.

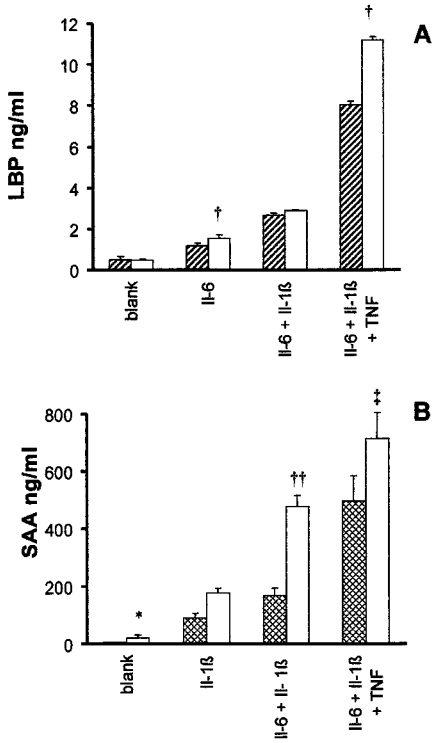

FIGURE 6. Dexamethasone enhances cytokine-induced LBP (*A*) and SAA (*B*) release by Caco-2 cells. Cells were stimulated for 72 h with IL-1β (5 ng/ml), IL-6 (100 ng/ml), TNF-α (100 ng/ml), or combinations of these agents in the presence (open bars) or the absence (hatched bars) of 1 μM dexamethasone. Controls consisted of unstimulated cells. After 72-h incubation, supernatants were collected, and LBP and SAA release was determined by ELISA. The data from a representative experiment are shown and expressed as the mean $\pm$ SD ($n = 6$). $*$, Significant positive effect of the individual parameter dexamethasone ($p < 0.01$); †, significant positive interaction between dexamethasone and cytokines ($p < 0.05$); ††, significant positive interaction between dexamethasone and cytokines ($p < 0.001$); ‡, significant negative interaction between dexamethasone and cytokines ($p < 0.001$).

LBP and SAA release by the intestinal cell lines Colo-205, T-84, and Int-407

To elucidate whether the release of LBP and SAA is restricted to Caco-2 cells or is a common feature of IEC, the capacities of three other intestinal epithelial cell lines to release LBP and SAA were evaluated. For this purpose Colo-205 and T-84 cells, both derived from the human colon, and Int-407 cells, derived from human ileum/jejunum, were cultured in the presence of IL-1β (5 ng/ml), IL-6 (100 ng/ml), TNF-α (100 ng/ml), and 1 μM dexamethasone. LBP release was inducible by cytokines in all tested cell lines. Nonstimulated cells produced only minute amounts of LBP. SAA was only detectable in the supernatant of stimulated Int-407 cells (Table I). These data confirm the capacity of human intestinal epithelial cells to synthesize and release LBP.

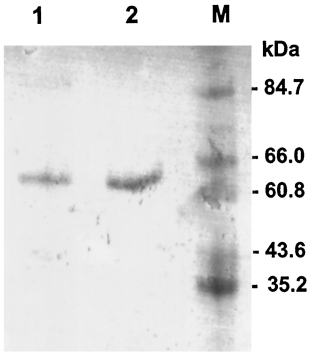

FIGURE 7. Western blot of the Caco-2-derived LBP and rLBP. LBP was isolated from the supernatant of stimulated Caco-2 cells and CHO-cells. Gel electrophoresis of LBP was followed by electrophoretic transfer onto an Immobilon-P membrane. LBP was detected by a polyclonal Ab to human LBP. *Lane 1*, rLBP; *lane 2*, LBP purified from supernatant of stimulated Caco-2 cells; *lane M*, molecular mass marker (kilodaltons).

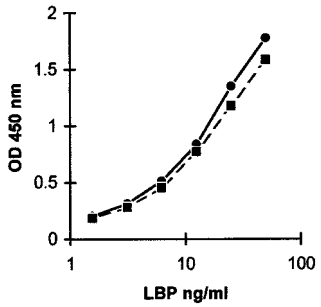

FIGURE 8. LPS binding capacity of Caco-2-derived LBP (●) and rLBP (■). Concentration range of Caco-2-derived LBP and rLBP added to plates coated with anti-LBP mAb HM14. Binding of biotinylated LPS to the immobilized LBP was determined. Data are expressed as OD 450 nm.

Discussion

In the present study we demonstrated the synthesis and release of the endotoxin binding protein LBP by human intestinal epithelial cells. Furthermore, the regulation of LBP and SAA release by IEC was studied in detail and compared with the regulation in liver cells. The results support the role of the intestinal mucosa as an active participant in the inflammatory response.

The results reveal that Caco-2, Colo-205, T-84, and Int-407 cells produce LBP. To our knowledge, this is the first evidence for the production of LBP by human IEC. Investigation of the induction of LBP release revealed that IL-6 induced LBP release by Caco-2 cells, while IL-1β and TNF-α showed a synergistic effect when used together with IL-6. Similarly, LBP synthesis was induced in liver cells by IL-6, whereas the effects of IL-1β and TNF-α on IL-6-induced LBP release were additive. TNF-α down-regulated the LBP synthesis induced by a combination of IL-1β and IL-6 in liver cells, whereas the opposite effect was found in Caco-2 cells. The data concerning the release of LBP by liver cells are supported by other reports (1, 21, 22).

SAA synthesis by Caco-2 cells was predominantly regulated by IL-1β, and this induction was enhanced by IL-6. This regulation is consistent with the SAA synthesis in liver cells, which is also

Tabel I: *LBP and SAA release by intestinal epithelial cell lines*[a]

		LBP (ng/ml)	SAA (ng/ml)
Colo-205	Unstimulated	1.05 $\pm$ 0.06	ND
	Stimulated	3.69 $\pm$ 0.10[b]	ND
T-84	Unstimulated	0.09 $\pm$ 0.09	ND
	Stimulated	0.69 $\pm$ 0.13[b]	ND
Int-407	Unstimulated	0.24 $\pm$ 0.38	ND
	Stimulated	0.85 $\pm$ 0.04[b]	410.6 $\pm$ 167.5[b]

[a] Cells were stimulated in the presence of IL-1β (5 ng/ml), IL-6 (100 ng/ml), TNF-α (100 ng/ml), and 1 μM dexamethasone for 72 h. Data are expressed as means $\pm$ SD. ND, not detectable.

[b] Significantly different from unstimulated cells ($p < 0.05$).

primarily regulated by IL-1β and shows strong synergism with IL-6 (23). The regulation of SAA synthesis by TNF-α in Caco-2 cells was shown to differ substantially from the regulation of SAA release by liver cells. TNF-α down-regulated the IL-1β-induced SAA release by HuH-7 cells, while in Caco-2 cells IL-1β and TNF-α showed an additive effect on SAA release. Subsequently, parallel to the differences seen for LBP induction between these cell types, TNF-α suppressed the SAA release induced by the combination of IL-6 and IL-1β in liver cells, while a synergistic effect was seen in Caco-2 cells. In summary, both proteins behave like secretory class 1 acute phase proteins in IEC, similar to their regulation in hepatocytes (19, 21, 23, 24), although differences in the regulation of LBP and SAA release between both cell types were discernible. These differences possibly reflect a distinction between the regulation of systemic levels, controlled by the liver, and the regulation of local tissue levels in the intestine, for which epithelial cells are responsible. The high levels of cytokines found in the intestine during intestinal inflammation (25, 26) seem in line with our hypothesis of a localized regulation of acute phase protein synthesis in the gut. Furthermore, the ratio of SAA to LBP secreted by Caco-2 cells is high compared with that in HuH-7 cells. It is not possible to conclude from the data that the absolute amount of SAA secreted by IEC exceeds the secretion of SAA by liver cells, because the numbers of Caco-2 and HuH-7 cells per well were not identical during experiments. However, these data clearly show a dramatic increase in SAA synthesis by Caco-2 cells in response to cytokines, which suggests that the intestinal mucosa is an important extrahepatic production site of SAA and which may reflect an immediate local requirement for this apolipoprotein during inflammation.

In contrast to cytokines, endotoxin induced neither LBP nor SAA release by Caco-2 cells in the presence of soluble CD14. The lack of response to endotoxin in Caco-2 cells is described for other acute phase proteins as well (8). Hypothetically this absence of response protects the host from an ongoing stimulation by endotoxins present in the gut lumen. Our data indicate that an inflammatory reaction leading to cytokine release is a necessary step for the induction of LBP and SAA release by IEC. Endotoxin enters under pathological circumstances the lamina propria, where it causes inflammation and the release of cytokines such as IL-6, IL-1, and TNF. We presume that these cytokines induce the release of acute phase proteins by the neighboring epithelium. However, it has been described that IEC themselves can generate cytokines in response to an endotoxin or bacterial challenge (27), although no consensus has been achieved in the literature concerning this property of IEC (8).

Glucocorticoids were demonstrated to enhance the cytokine-mediated induction of LBP and SAA release by IEC. This result is consistent with the enhancing effect of glucocorticoids on cytokine-induced SAA (24) and LBP (19, 21) synthesis by liver cells, as reported previously and confirmed in this study (data not shown). The data indicate that the local synthesis of acute phase proteins in the gut is reinforced by glucocorticoids, which are synthesized by the adrenal glands in high amounts during a systemic acute phase response.

We hypothesize that the release of LBP by human intestinal epithelial cells is involved in the defense against endotoxins, which is pre-eminently important in the mucosa. Besides its role in neutralization and clearance of endotoxin, a basal concentration of LBP is known to enhance the sensitivity of the immune system to endotoxin by catalyzing the binding of endotoxin to macrophages. LBP produced by IEC may therefore also have a function in sensitizing the mucosal immune system to translocated endotoxins. However, high concentrations of LBP have been shown to decrease endotoxin activity (4). The ratio of LBP to endotoxin is reported to determine a number of essential processes: the monocytic response (4), the binding of LBP to HDL (28), and the inactivation of endotoxin by HDL (29). We suggest that local production of LBP by epithelial cells during inflammation in the intestine may result in high local concentrations of LBP and consequently to a reduced toxicity of endotoxin. The proposed importance of LBP in local defense against Gram-negative bacteria is supported by recent studies demonstrating that i.p. administration of bacteria is tolerated by wild-type mice, although it results in uncontrolled multiplication and spread of bacteria in LBP knockout mice (30). Moreover, i.p. injection of LBP can reduce mortality mediated by endotoxin and bacteria in mice (4).

Secretion of SAA by the intestinal epithelium refers to a role for SAA in the local inflammatory processes in the intestine. Although the primary function of SAA in the acute phase response is not fully understood, several immunological functions have been proposed. There is evidence that SAA enhances the binding of HDL, which possesses substantial endotoxin binding capacity (2), to macrophages (9). Furthermore, it was demonstrated recently that SAA has cytokine-like properties (31). Further studies are necessary to determine the biological role of SAA secretion by the intestinal mucosa.

In summary, we have demonstrated the release of LBP and SAA by IEC in response to specific sets of cytokines and glucocorticoids. Endotoxin is shown not to induce the release of LBP and SAA by IEC, indicating that cytokine release in the proximity of the epithelial cells is necessary for the induction of LBP and SAA release by these cells. The capability of IEC to release LBP and SAA suggests a role for the intestinal epithelium in the acute phase response and the local defense of the gut against bacteria and endotoxin.

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