Endotoxemia in mice stimulates production of complement C3 and serum amyloid A in mucosa of small intestine

QUAN WANG, TORY A. MEYER, STEVEN T. BOYCE, JING JING WANG, XIAOYAN SUN, GREG TIAO, JOSEF E. FISCHER, AND PER-OLOF HASSELGREN
Department of Surgery, University of Cincinnati, and the Shriners Hospital for Children, Cincinnati, Ohio 45267-0558

Wang, Quan, Tory A. Meyer, Steven T. Boyce, Jing Jing Wang, Xiaoyan Sun, Greg Tiao, Josef E. Fischer, and Per-Olof Hasselgren. Endotoxemia in mice stimulates production of complement C3 and serum amyloid A in mucosa of small intestine. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1584–R1592, 1998.—We examined the effect of endotoxemia in mice on protein and mRNA levels for the acute phase proteins complement C3 and serum amyloid A (SAA) in jejunal mucosa. Endotoxemia was induced in mice by the subcutaneous injection of 250 μg lipopolysaccharide per mouse. Control mice were injected with saline. C3 and SAA were measured by ELISA. Messenger RNA levels were determined by Northern blot analysis or competitive PCR. Immunohistochemistry was performed to determine in which cell type(s) C3 and SAA were present. Mucosal C3 and SAA protein and mRNA levels were increased in endotoxemic mice. Immunohistochemistry showed that C3 was present in both enterocytes and cells of the lamina propria, whereas SAA was seen mainly in lamina propria cells. Results suggest that endotoxemia stimulates production of C3 and SAA in small intestinal mucosa. The response may be regulated at the transcriptional level and probably reflects increased synthesis of the acute phase proteins in both enterocytes and cells of the lamina propria.

MATERIALS AND METHODS

Animals and experimental design. Male A/J (20–27 g) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed at a temperature of 22°C in a room with a 12:12-h light-dark cycle for 1 wk before experiments. Endotoxia was induced by the subcutaneous injection of 250 μg/mouse of lipopolysaccharide (Escherichia coli 0111:B4; Calbiochem, LaJolla, CA). Control mice were injected with corresponding volumes of sterile saline. Food was withheld, but drinking water was provided after the injection of endotoxin or saline. The dose of endotoxin used here was based on a recent report in which we found that the subcutaneous injection of the same amount of endotoxin in mice gave rise to a maximal increase in total mucosal protein synthesis in the jejunum (31). The endotoxin was injected subcutaneously rather than intraperitoneally to avoid the influence of local metabolic changes when intestinal mucosa was studied. Groups of mice were studied at intervals up to 24 h after injection of endotoxin or saline. With mice under pentobarbital sodium anesthesia (40 mg/kg ip), blood was collected by heart puncture in heparinized tubes for determination of plasma concentrations of C3 and SAA. We harvested the mucosa from a 10-cm segment of the jejunum by opening the intestine along the antimesenteric border and by scraping the luminal side with a microscope slide. The mucosa was immediately frozen in liquid nitrogen and stored at −70°C until analysis. In a separate series of experiments, the superior mesenteric artery was catheterized and slowly perfused with 0.5–1 ml of saline before harvesting of the intestinal mucosa to minimize the contribution of acute phase proteins from the blood to the acute phase proteins that were measured in the tissue. The gut; enterocyte
intestinal wall was noted to blanch before mucosa was harvested. All experiments were performed, and the animals were cared for according to the National Research Council's "Guide for the Care and Use of Laboratory Animals." The experimental protocol was approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Measurement of complement C3 and SAA. Plasma and tissue levels of complement C3 and SAA were measured by ELISA. For determination of complement C3 and SAA in mucosa and liver, tissue was ultrasonicated two times for 10 s in 1 ml of PBS containing 2 µg/ml each of the protease inhibitors leupeptin, aprotinin, pepstatin A, and antipain (Sigma, St. Louis, MO) and 2 mM phenylmethylsulfonyl fluoride (Sigma) and then centrifuged at 12,000 g at 4°C for 45 min. The supernatants were used for determination of complement C3 and SAA. Complement C3 was determined as described previously (13) using a goat anti-mouse C3 antibody (IgG fraction 55463; Cappell Research Products, Durham, NC). SAA was measured with a commercially available ELISA kit using a rat anti-human SAA antibody (Biosource International, Camarillo, CA). This antibody has a 100% cross-reactivity to mouse SAA according to the manufacturer. The lower limits of detection were 10 ng/ml for complement C3 and 0.23 µg/ml for SAA. One concern when measurements of C3 levels are performed is the specificity of the anti-C3 antibody, i.e., whether the antibody recognizes the cleavage products of C3 as well. In a control experiment using purified mouse C3 and purified human C3, C3a, C3b, and C3bi (purified mouse C3a, C3b, and C3bi are not commercially available), we found evidence that the anti-mouse C3 anti-body used here recognizes C3, C3b, and C3bi but not C3a. Thus the results obtained with this antibody may reflect the presence both of C3 and of some of its cleavage products.

mRNA for complement C3 and SAA. Northern blot analysis and competitive PCR were used to determine the expression of mucosal and hepatic C3 and SAA mRNA. Mucosal samples were harvested as described in Measurement of complement C3 and SAA, and samples from two or three mice were pooled for each time point. Total RNA was extracted by the guanidinium thiocyanate-phenoI-chloroform method (5) using a Stat-60 kit (Tel-Test B, Friendswood, TX).

For Northern blot analysis, RNA was denatured and separated by electrophoresis on a 1% agarose gel containing formaldehyde. The RNA was transferred from the gel to nylon membranes (Micron Separations, Westboro, MA) by capillary action in 2× saline sodium citrate (SSC) (1× SSC = 0.15 M NaCl and 15 mM sodium citrate) overnight. RNA was immobilized either by baking at 80°C for 2 h or by ultraviolet cross-linking. The blots were hybridized at 42°C for 4 h in 50%formaldehyde and 6× sodium chloride-sodium phosphate-EDTA (SSPE) (1× SSPE = 0.15 M NaCl, 10 mM NaH2PO4 and 1 M EDTA), 5× Denhardt's solution, 0.5% SDS, and 100 µg/ml salmon sperm DNA. CDNA probes for C3 and SAA were labeled by random priming with [32P]dATP or [35P]dCTP (Stratagene, La Jolla, CA). The blots were hybridized with the [32P]-labeled cDNA probes at 42°C overnight. The blots were then washed two times in 1× SSC and 0.1% SDS and one time in 0.1× SSC and 0.1% SDS at room temperature and autoradiographed at −70°C. The blots were stripped and rehybridized with an 185 oligonucleotide probe to control for equal loading of RNA.

Competitive PCR was performed as described in detail previously (26). In short, total RNA was extracted from mucosa as described in mRNA for complement C3 and SAA. Dissolved total RNA samples were reverse transcribed to cDNA using a Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI). Briefly, 5 µl of total RNA samples were added to 1 µl 3'-primer in 50 mM Tris·HCl (pH 7.3) and heated to 90°C following by cooling to 37°C over 15 min. Reaction buffer, 75 mM KCl, 3 mM MgCl2, 20 mM dithiothreitol, 50 U RNasin, 35 U DNase, 250 µM dNTP, and 250 U M-MLV RT were then added, and the samples were incubated at 37°C for 40 min, followed by freezing at −70°C for at least 15 min to inactivate the enzyme. The resulting cDNA samples were stored at −70°C until used. Aliquots of the cDNA samples were then added to a cocktail containing PCR buffer, gelatin, KCl, MgCl2, dNTP, C3 primers, and Taq polymerase (Promega). A sequence of 385 nt in mouse complement C3 mRNA (exons 4-7) (7) was used to construct the C3 primers: 5'-sense 5'-CAC CGC AAA TGC CTA C-3' (nt 2684-2703) and 3'-antisense 5'-GAT CAG GTG TTT CAG CCG C-3' (nt 3049-3068). PCR was performed using a Perkin-Elmer DNA thermocycler by heating the samples to 95°C for 2 min, followed by 25 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 2 min. The samples were kept at 60°C for 7 min and cooled to 4°C. Ten microliters of the PCR products were then separated by electrophoresis at 100 V in a 1.5% agarose gel stained with ethidium bromide. To quantify mRNA expression, a PCR MIMIC construction kit (Clontech, Palo Alto, CA) was used to construct homologous DNA fragments using the mouse C3 gene-specific primer and control primers. An initial PCR amplification using the composite primers was performed on the neutral DNA fragment that incorporated the C3 gene-specific primer sequence onto the neutral DNA. The yield of this PCR MIMIC was calculated as the ratio between the optical densities at 260 and 280 nm and diluted to 100 attomol/µl. Competitive PCR was then performed by adding 2 µl of the cDNA samples to 2 µl of different 10-fold dilutions of PCR MIMIC (100–10−6 attomol), PCR buffer, MgCl2, nucleotide triphosphates (Gibco-BRL, Grand Island, NY), 5' and 3'-C3 gene-specific primers, and Taq polymerase (Promega). The mixture was heated to 94°C for 45 s, to 50°C for 45 s, and to 72°C for 90 s. The samples were kept at 72°C for 7 min and cooled to 4°C. Five microliters of the PCR products were then separated by electrophoresis at 100 V in a 1.5% agarose gel stained with ethidium bromide. Comparison between PCR MIMIC bands (known concentrations) and C3 bands was made to determine the concentration of C3 mRNA present using a video image system (BioMax 1D Image Analytic System; Eastman Kodak, Rochester, NY).

Immunohistochemistry. Immunohistochemistry was performed to determine in which cell type(s) of the mucosa complement C3 and SAA were present. With mice under pentobarbital anesthesia, the lumen of the jejunum was flushed with saline and a 0.5-cm segment was excised from the midportion of the jejunum. The intestinal segment was immersed in OCT embedding medium (Miles, Elkhart, IN) and then placed on a metal board frozen in liquid nitrogen. The samples were stored at −70°C until analysis.

Five-micron sections were cut and embedded on poly-l-lysine-coated slides. The staining was performed as described previously (9). The sections were dried at 45°C for 10 min and fixed in 100% methanol at −20°C for 10 min. The sections were then blocked with 0.1% avidin (no. A-9275, Sigma) and 0.1% b-biotin (no. B-4501) for 15 min each and further blocked with 5% BSA for 30 min at room temperature. The primary antibodies, which were the same as used for the ELISA assays described but diluted 1:100, were added to the sections, which were then incubated for 2 h at 37°C. Biotin conjugated rabbit anti-goat IgG (no. 39559, Cappell Research Products) and fluorescence conjugated streptavidin (no. 1055097; Boehringer, Mannheim, Germany) were diluted 1:2,000 and used consecutively for further detection of C3.
Goat anti-rat IgG conjugated with biotin (no. 55790, Cappel Research Products) was used as secondary antibody for detection of SAA. During staining, Tris-buffered saline (0.05 M, pH 7.6) was used as the buffering system throughout and each step was followed by washes. Negative controls were performed by omitting the primary antibody. The sections were examined with a Nikon microphot-FXA (Nikon, Melville, NY) equipped with an epifluorescence illumination system, and photographs were observed on Ektachrome 400 ASA professional daylight film. Note that when the anti-mouse C3 antibody was used, immunohistochemistry may reflect the presence of C3 as well as its cleavage products C3b and C3bi, as described in Measurement of complement C3 and SAA.

Statistical analysis. Results are expressed as means ± SE. The Mann-Whitney rank sum was used for statistical analysis.

**RESULTS**

Complement C3 levels in jejunal mucosa were increased by 50 and 86% 16 and 24 h after injection of endotoxin, respectively (Fig. 1A). Complement C3 concentrations in liver were five to six times higher than those in mucosa and were increased in endotoxemic mice at 8 h and through the rest of the experimental period (Fig. 1B). Plasma levels of C3 were increased 16 and 24 h after injection of endotoxin (Fig. 1C). Because the anti-mouse C3 antibody used in these experiments may recognize C3b and C3bi in addition to C3, the results may reflect the sum of C3 and its cleavage products C3b and C3bi. For clarity, results are described as C3 throughout the rest of the paper, but it is important to recognize this limitation of the study.

Endotoxemia resulted in an approximately fourfold increase in mucosal SAA levels at 16 and 24 h (Fig. 2A). SAA concentrations were higher in liver than in mucosa and responded to endotoxemia in a similar way, with increased levels noted 16 and 24 h after injection of endotoxin (Fig. 2B). As expected (29), plasma concentrations of SAA were markedly elevated in endotoxemic mice, with a more than 20-fold increase noted at 16 h (Fig. 2C). Interestingly, plasma levels of SAA were increased already 4 and 8 h after injection of endotoxin, i.e., before the increase in mucosal and liver concentrations took place.

Because it is possible that blood-borne acute phase proteins, deposited in the tissue, could have caused or at least contributed to the increased mucosal levels noted at 16 and 24 h in endotoxemic mice, a control experiment was performed in which the intestinal vasculature was perfused with saline before the mucosa was harvested. In this experiment, the basal C3 levels in the mucosa were somewhat lower than in mucosa of intestine in which the vasculature had not been perfused with saline, whereas the basal levels of SAA were not reduced by the perfusion (Fig. 3B; compare with Fig. 2A). To examine whether the increased mucosal complement C3 and SAA levels during endotoxemia reflected an increased local production of the proteins, possibly regulated at the transcriptional level, we next measured mucosal levels of mRNA for C3 and SAA. When Northern blot analysis was performed, no signals for C3 or SAA mRNA were noticed in samples from saline-injected control mice. C3 mRNA was not detected on Northern blots in mucosal samples of endotoxemic mice but was present in liver tissue, with a faint band noticed at 2 h and a maximal response noticed 16 h after injection of endotoxin (Fig. 4). SAA mRNA was induced in jejunal mucosa of endotoxemic mice, with a
weak signal noticed at 4 h after injection of endotoxin and a maximal response seen at 16 h (Fig. 5). In liver, the expression of SAA mRNA was induced already 2 h after injection of endotoxin and was sustained during the rest of the 24-h experimental period (Fig. 6). Note that in liver, two bands were present on Northern blots, similar to a recent report by de Beer et al. (6). The two mRNA bands reflect the fact that SAA is a multigene family; the heavier band (between 18S and 28S) may correspond to SAA5, and the lighter mRNA (below 18S) may correspond to SAA2 (6). In jejunal mucosa, only one band for SAA mRNA was seen (Fig. 5) and, on the basis of previous reports (6, 18), this band may represent mRNAs for SAA1–3.

Because the absence of a clear mucosal signal for C3 mRNA on the Northern blots could be due to insufficient amounts of C3 mRNA in the mucosa, we performed PCR on RNA extracted from jejunal mucosa. Results from that experiment showed that mRNA for complement C3 was detectable in jejunal mucosa of both control and endotoxemic mice. To test whether endotoxemia resulted in increased C3 mRNA levels, competitive PCR was performed. Results from that experiment revealed an ~100% increase in mucosal C3 mRNA levels 4 h after induction of endotoxemia and a
five- to sixfold increase at later time points (Fig. 7A). Because of the high sensitivity of PCR, one concern was that the signal for C3 mRNA noticed in mucosa may reflect “contamination” by nonmucosal cells. A control experiment was therefore performed in which mucosa was harvested after perfusion of the intestinal vasculature at different time points after saline or endotoxin injection. Competitive PCR showed an almost identical response to endotoxin as the one seen in the experiment in which the vasculature was not perfused (Fig. 7B; compare with Fig. 7A). Also, the basal levels of C3 mRNA were similar in the two experiments, suggesting that the C3 mRNA levels reflected local tissue mRNA rather than mRNA in circulating cells.

Although the results described suggest that mucosal levels of C3 and SAA are increased during endotoxemia and that the increased protein levels may reflect transcriptional upregulation of the protein, the cellular origin of the proteins is not known. To test in which cell type(s) of the mucosa complement C3 and SAA were present, we performed immunohistochemical studies.

Fig. 5. Northern blot analysis of RNA extracted from jejunal mucosa of endotoxemic (LPS) and saline-injected control mice. Blots were hybridized with a cDNA probe for SAA, stripped, and rehybridized with an 18S oligonucleotide probe to control for equal loading of RNA.

Fig. 6. SAA mRNA levels in livers of endotoxemic and saline-injected control mice determined by competitive PCR. Groups of mice were studied 1, 4, 8, and 16 h after subcutaneous injection of 250 µg/mouse of endotoxin or a corresponding volume of saline; n = 3 per group at each time point.

C3 was present in both enterocytes and cells of the lamina propria (Fig. 8), whereas SAA was expressed mainly in cells of the lamina propria (Fig. 9). In endotoxemic mice, SAA was detected in the intestinal lumen (Fig. 9C). Evidence of mucosal injury was seen in the endotoxemic mice, with disruption of the epithelial layer and desquamation of cells into the intestinal lumen (Fig. 8, C and D and Fig. 9, C and D).

DISCUSSION

In the present study, endotoxemia in mice was associated with increased concentrations of the acute phase proteins complement C3 and SAA in jejunal mucosa. Because C3 and SAA mRNAs were upregulated by
endotoxin and immunohistochemistry indicated that the proteins were expressed in both enterocytes and nonepithelial mucosal cells; results are consistent with the concept that the local production of C3 and SAA may be increased in mucosa of small intestine during endotoxemia. The results are important because they support the role of the intestinal mucosa in the metabolic response to sepsis and endotoxemia and provide further evidence that the gut is an active participant in the inflammatory response to these conditions.

One major concern when the present results were interpreted was whether the mucosal levels of C3 and SAA represented local production of the proteins or merely a deposition or contamination of blood-borne proteins. Although circulating C3 and SAA probably contributed to the mucosal levels, several lines of evidence suggest that local production of the proteins took place in the mucosa. 1) When the vasculature of the jejunum was perfused with saline, the difference in mucosal C3 and SAA levels between control and endotoxemic mice persisted; 2) the increased mRNA levels for C3 and SAA, in particular the persistent increase in C3 mRNA after perfusion of the intestinal vasculature, strongly support a local production of the proteins; 3) when immunohistochemistry was applied, results showed that C3 was present in enterocytes and other mucosal cells and SAA was present in nonepithelial mucosal cells; and 4) there is evidence that enterocytes can synthesize several acute phase proteins, including C3 and SAA (2, 3, 23, 24, 27). Thus the increased mucosal levels of complement C3 and SAA may at least in part reflect increased local production of the proteins during endotoxemia.

To our knowledge, this is the first report of increased mucosal production of C3 and SAA during endotoxemia. In a recent report, increased SAA mRNA levels were noticed in intestine of endotoxemic mice but SAA protein levels were not measured (6). In other studies, mucosal production of acute phase proteins was increased in inflammatory bowel disease (1, 11, 12). In previous experiments, Molmenti et al. (23) found evidence for an acute phase response in human intestinal
epithelial cells. In recent experiments in our laboratory, complement C3 protein and mRNA were expressed in cultured Caco-2 cells, a human intestinal epithelial cell line (23). Thus the intestinal mucosa may be the production site of acute phase proteins, both in response to local inflammation, such as inflammatory bowel disease (1, 11, 12), and in response to systemic inflammation, such as endotoxemia (present results).

Although the present report and other studies (23, 27) suggest that acute phase proteins may be produced at extrahepatic sites, the liver is probably the major site of acute phase protein synthesis, at least from a quantitative standpoint. In the present study, the concentrations of C3 and SAA were four to five times higher in liver tissue than in mucosa, supporting the central role of the liver in the acute phase response (29). The present finding of elevated circulating levels of SAA in endotoxemic mice before liver and mucosal levels were increased was surprising and may suggest that other tissues as well are involved in the acute phase response. Alternatively, the breakdown, excretion, and/or tissue distribution of circulating SAA was influenced by endotoxin before changes in liver and mucosal levels occurred.

The influence of endotoxemia on mucosal complement C3 levels was examined in the present study because C3 may be particularly important in the mucosa. Complement C3 converges the classical and alternative pathways into a final common pathway in the complement cascade which participates in the local defense against invading microorganisms and may cause lysis of bacteria (8). SAA was studied because it is the major acute phase protein in mice (4, 10, 29). Although the exact biological role of SAA in the acute phase response is unidentified, SAA is an apoprotein of HDL and may influence the formation and clearance of HDL (17). It should be noted that murine SAA is encoded by multiple genes and that at least five members of the SAA family have been identified and labeled SAA1 through SAA5 (6, 18, 20). The SAA proteins are highly homologous proteins (32), and the antibody used in the present study for ELISA and immunohistochem...

Fig. 9. Immunohistochemistry of jejunal mucosa from saline-injected (A) and endotoxemic (C) mice showing presence of SAA mainly in cells of lamina propria. Negative controls were performed by omitting primary SAA antibody (B and D). Light green areas in A and C represent signal for SAA. Mucosa was harvested 24 h after injection of endotoxin or saline. Bar in B, 100 µm.
The intestinal mucosa and increased synthesis of SSA and SAA in liver. Likely represented upregulated production of SAA1–3 in reports of the expression of different members of the SAA family. The present results support the concept that the intestinal mucosa is an active participant, rather than only a passive bystander, in the metabolic and inflammatory response to sepsis and endotoxia. Although the majority of the acute phase proteins are probably produced in the liver, the observations in this study are important because they suggest that the intestinal mucosa may contribute to the acute phase response. It should be an important focus for future studies to determine the biological role of mucosal acute phase proteins. It is likely that they are most important for the local metabolic response in the intestinal mucosa. Because mucosal acute phase proteins may be secreted into the portal vein, it is also possible that they can regulate the hepatic response to sepsis and endotoxia.

Perspectives

The present results support the concept that the intestinal mucosa is an active participant, rather than only a passive bystander, in the metabolic and inflammatory response to sepsis and endotoxia. Although the majority of the acute phase proteins are probably produced in the liver, the observations in this study are important because they suggest that the intestinal mucosa may contribute to the acute phase response. It should be an important focus for future studies to determine the biological role of mucosal acute phase proteins. It is likely that they are most important for the local metabolic response in the intestinal mucosa. Because mucosal acute phase proteins may be secreted into the portal vein, it is also possible that they can regulate the hepatic response to sepsis and endotoxia.


