Effects of *Anaplasma phagocytophilum* on Host Cell Ferritin mRNA and Protein Levels

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Received 11 May 2005/Returned for modification 13 July 2005/Accepted 22 July 2005

Ferritin is a major intracellular iron storage protein and also functions as a cytoprotectant by sequestering iron to minimize the formation of reactive oxygen species. *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis, is an obligate intracellular bacterium that colonizes neutrophils. We have previously reported that human promyelocytic HL-60 cells infected with *A. phagocytophilum* demonstrate increased transcription of ferritin heavy chain and also that the bacterium stimulates neutrophil NADPH oxidase assembly and degranulation during the initial hours of infection (J. A. Carlyon, W. T. Chan, J. Galan, D. Roos, and E. Fikrig, J. Immunol. 169:7009–7018, 2002, and J. A. Carlyon, D. Abdel-Latif, M. Pypaert, P. Lacy, and E. Fikrig, Infect. Immun. 72:4772–4783, 2004). In this study, we assessed ferritin mRNA and protein levels during *A. phagocytophilum* infection in vitro using HL-60 cells and neutrophils and in vivo using neutrophils from infected mice. The addition of *A. phagocytophilum*, as well as *Escherichia coli* and serum-opsonized zymosan, to neutrophils results in a pronounced increase in ferritin light-chain transcription and a concomitant rise in ferritin protein levels. Neutrophils from *A. phagocytophilum*-infected mice demonstrate elevated ferritin heavy-chain mRNA expression, a phenomenon consistent with infections by intracellular pathogens. Notably, ferritin protein levels of infected HL-60 cells were markedly diminished in a dose- and time-dependent manner. These studies provide insight into the effects *A. phagocytophilum* has on the ferritin levels of its host cell.

*Anaplasma phagocytophilum* is the etiologic agent of human granulocytic anaplasmosis, an emerging disease that is gaining increased recognition in the United States, Europe, and Asia. *A. phagocytophilum* is an obligate intracellular bacterium that naturally exists in a zoonotic cycle between ticks of the *Ixodes persulcatus* complex and small mammals. Humans are accidental hosts. The bacterium resides within the salivary glands of its arthropod vector and, following inoculation into a mammalian host, preferentially colonizes neutrophils or neutrophil precursors (15, 16, 19). Nonspecific symptoms associated with human granulocytic anaplasmosis include fever, chills, headache, malaise, and myalgia. More distinguishing manifestations consist of leukopenia, thrombocytopenia, and elevated levels of C-reactive protein and hepatic transaminases. Though usually self-limiting, severe complications can result and include prolonged fever, shock, seizures, pneumonitis, acute renal failure, hemorrhages, rhabdomyolysis, and death (3, 15–17).

Bacterial pathogens can freely acquire necessary nutrients from their hosts, except for iron, which is essential for the growth of nearly all bacterial species (37, 45). The concentration of free iron in mammals, however, is exceptionally low, because it is sequestered by iron-withholding proteins. Maintenance of proper intracellular iron levels involves its uptake, utilization, and storage. Extracellular iron is bound by transferrin, which docks to its receptor, is endocytosed, and releases the iron before recycling back to the cell surface (24, 42). The delivered iron becomes part of the labile iron pool (LIP), which can either be used immediately for metabolic processes or stored (23, 47, 49). It is crucial that proper iron homeostasis be maintained, as excess iron provides a source for the growth of intracellular bacteria (37, 42). Furthermore, excess levels promote formation of cytotoxic reactive oxygen species (ROS). Excess iron from the LIP can participate in the Fenton reaction [Fe(II) + H₂O₂ → Fe(III) + OH⁻ + OH⁻], which yields a hydroxyl radical, the most potent oxidant in biological systems (47). The toxicity of iron is largely attributable to its capacity to generate such reactive species.

Ferritin is the major intracellular iron storage protein of prokaryotes and eukaryotes (23, 47). Mammalian ferritin is a 24-mer protein composed of various proportions of heavy (H) and light (L) subunits and has an extremely high storage capacity of up to 4,500 iron atoms. Steps in iron storage within ferritin molecules consist of Fe(II) oxidation, Fe(III) migration, and iron core formation. H chains are important for Fe(II) oxidation, while L chains assist in core formation (23). Ferritin molecules with greater proportions (≥67 to 83%) of ferritin light chains (FLC) are most efficient at incorporating and storing iron (23, 47). By removing excess iron from the LIP, ferritin also serves as a cytoprotectant against ROS-mediated damage (4, 11, 21, 26, 30–32, 39, 50). Indeed, ferritin levels increase as a direct response to oxidative stress. Other factors that regulate ferritin expression include intracellular iron concentration, gamma interferon (IFN-γ), proinflammatory cytokines, growth factors, and differentiation (47).

We have previously shown that *A. phagocytophilum* infection of HL-60 cells and all-*trans* retinoic acid-differentiated HL-60...
Peripheral blood from healthy donors by centrifugation through an equal volume of (Columbus).

Strain HZ was kindly provided by Ralph Horowitz of New York scid inbred C3H-scid-infected C3H-scid mice. The cells were centrifuged at 210

A. phagocytophilum p44 F1
A. phagocytophilum p44 R1
β-actin F1
β-actin R1
fhc F1
fhc R1
fhc QR1
fhc Taqman probe
m-hprt F1
m-hprt R1
m-fhc F1
m-fhc R1
m-flc F1
m-flc QR1

A. phagocytophilum binding and invasion of neutrophils in vitro stimulates NADPH oxidase assembly and degranulation, two processes that result in ROS release (12). Because ferritin could serve as a major cytoprotectant against oxidative stress, we further investigated the effects of A. phagocytophilum on ferritin mRNA and protein expression in HL-60 cells and human neutrophils during in vitro infection and in murine neutrophils during in vivo infection. These studies shed light onto the influence of *A. phagocytophilum* on the ferritin levels of its mammalian host cell.

**MATERIALS AND METHODS**

**Cultivation of *A. phagocytophilum* and maintenance of infection.** *A. phagocytophilum* strains NCH-1 and HZ were cultivated in HL-60 cells as previously described (12). At 1:3, and 6 h postinfection, either total RNA or protein was isolated as described below.

**RNA isolation and reverse transcription-PCR (RT-PCR).** Total RNA from infected or *A. phagocytophilum*-infected cells was isolated using Trizol (Invitrogen) according to the manufacturer’s instructions followed by treatment with RNase-free DNase (1 U/μg; Roche Molecular Biochemicals, Indianapolis, IN) for 2 h at 37°C. DNA-free RNA was reconstituted using Trizol, ethanol precipitated, and resuspended in diethyl pyrocarbonate-treated water. cDNA stocks (50 μl) were prepared from 5 μg of total RNA using random hexamers and the ProSTAR First Strand RTPCR kit (Stratagene, La Jolla, CA), followed by PCR amplification using 1 μl of cDNA template. The thermal cycling conditions used were 94°C for 2 min followed by 35 cycles of 94°C for 60 s, 55°C for 1 min, and 72°C for 1 min. In some cases, zymosan A particles from *Saccharomyces cerevisiae* (Sigma) that had been opsonized with autologous serum (OpZ) or Escherichia coli were added to neutrophils as previously described (12). At 1, 3, and 6 h postinfection, either total RNA or protein was isolated as described below.

**Quantitative RT-PCR.** cDNAs generated from uninfected or *A. phagocytophilum*-infected cells were used as templates for quantitative PCR using the MyIQ Real Time Detection System (Bio-Rad, Richmond, CA). Each cDNA was in a reaction mixture that contained final concentrations of 1.5 U platinum Taq DNA polymerase (Life Technologies, Gaithersburg, MD), 20 nM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl2, 200 μM deoxynucleoside triphosphates, 0.8 μM of each primer, and 2.5 U of Taq polymerase (Roche Molecular Biochemicals). To ensure that RNA templates were free from contaminating DNA, identical reactions were performed in the absence of reverse transcriptase (data not shown). The primers used are listed in Table 1.

**Isolation of human neutrophils.** Human neutrophils were isolated from peripheral blood by centrifugation through an equal volume of Polymorphprep (Axis-Shield; Greiner Bio-One, Frickenhausen, Germany) at 470 × g for 30 min. The resulting neutrophil band was removed by aspiration and mixed with equal volumes of 0.45% NaCl and Iscove’s modifed Dulbecco’s medium (IMDM; Invitrogen, Carlsbad, CA)-0.5 mM EDTA to restore isotonic conditions. The cells were centrifuged at 210 × g for 10 min, and the supernatant was removed. The resulting pellet was resuspended and incubated in 10 μl of red blood cell lysing buffer (Sigma, St. Louis, Mo.) for 10 min followed by two successive washes in IMDM-0.5 mM EDTA and enumeration using a hemacytometer.

**In vitro *A. phagocytophilum* infection.** Host-cell-free *A. phagocytophilum* cells were recovered from infected HL-60 cells and added to 5 × 10⁶ HL-60 cells (0.5 × 10⁶/ml) in IMDM-10% fetal bovine serum. The ratios of *A. phagocytophilum* organisms per HL-60 cell were determined as described previously (12). For in vitro infection of human neutrophils, 5 × 10⁶ neutrophils (1 × 10⁶/ml) in IMDM were added to individual wells of either 6- or 24-well Ultra Low Attachment plates (Corning, Inc.; Corning, NY). To these were added suspensions of host-cell-free *A. phagocytophilum*. In some cases, zymosan A particles from *Saccharomyces cerevisiae* (Sigma) that had been opsonized with autologous serum (OpZ) or Escherichia coli were added to neutrophils as previously described (12).

**TABLE 1. Oligonucleotide primers used in this study**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. phagocytophilum p44 F1</td>
<td>5′-TCAGAGCCAAGGTTATAGATA-3′</td>
<td>Bases 395–419 (+strand)</td>
</tr>
<tr>
<td>A. phagocytophilum p44 R1</td>
<td>5′-GCCACTATGTTTTCCTCGG-3′</td>
<td>Bases 898–920 (−strand)</td>
</tr>
<tr>
<td>β-actin F1</td>
<td>5′-AGCGGGAATCGTGCGT-3′</td>
<td>Bases 614–631 (+strand)</td>
</tr>
<tr>
<td>β-actin R1</td>
<td>5′-CAGGGTACATGTTGGGC-3′</td>
<td>Bases 901–922 (−strand)</td>
</tr>
<tr>
<td>fhc F1</td>
<td>5′-CGACCGCTGCACCTCG-3′</td>
<td>Bases 5–21 (−strand)</td>
</tr>
<tr>
<td>fhc R1</td>
<td>5′-TTGAGTGTTTAGGATA-3′</td>
<td>Bases 504–521 (−strand)</td>
</tr>
<tr>
<td>fhc QR1</td>
<td>5′-TCACTACACAGTCAGG-3′</td>
<td>Bases 229–254 (+strand)</td>
</tr>
<tr>
<td>fhc Taqman probe</td>
<td>5′-ACGAGTGGGCGATATTTTTCCAGG-3′</td>
<td>Bases 5–21 (−strand)</td>
</tr>
<tr>
<td>m-hprt F1</td>
<td>5′-CGCCAGGCTCTACCTAC-3′</td>
<td>Bases 71–94 (+strand)</td>
</tr>
<tr>
<td>m-hprt R1</td>
<td>5′-TGACGTTAACACTCCGTAGT-3′</td>
<td>Bases 575–599 (+strand)</td>
</tr>
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<td>m-fhc F1</td>
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<td>Bases 514–529 (−strand)</td>
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<tr>
<td>m-flc QR1</td>
<td>5′-ATATTCTGCATGCGCAG-3′</td>
<td>Bases 16–36 (−strand)</td>
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<td>m-flc Taqman probe</td>
<td>5′-ACGAGGTGGCCGAATCTTCCTTCAGG-3′</td>
<td>Bases 479–495 (−strand)</td>
</tr>
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</table>

a m, murine; hprt, hypoxanthine ribosyltransferase.
Ferritin enzyme-linked immunosorbent assay (ELISA). Whole-cell lysates were examined for ferritin content using the Human Ferritin ELISA kit (Alpha Diagnostic, San Antonio, TX) according to the manufacturer’s instructions. Briefly, 30 µg of whole-cell lysate, 30 µg bovine serum albumin (BSA), or 10 µl of recombinant ferritin standards, ranging from 0 to 800 ng/ml, were added in duplicate to wells of a 96-well plate coated with anti-ferritin MAb. One-hundred microliters of horseradish peroxidase-conjugated anti-ferritin MAb were added, followed by a 30-min incubation at room temperature. The plate was washed three times with 300 µl deionized water, followed by the addition of 200 µl detection substrate per well. After 10 min, 50 µl stop solution was added and the absorbance at 450 nm was recorded using an AD 340 Absorbance Detector (Beckman Coulter, Fullerton, CA).

Assessment of fhc and flc mRNA expression during murine A. phagocytophilum infection. Infected blood from C3H/scid mice was used to initiate infection in immunocompetent C3H/HeN mice (3 per group) as previously reported (13). Nine percent of peripheral blood neutrophils in the inoculum had detectable morulae. Control groups were injected with uninfected C3H/scid mouse blood. On days 2 and 8, the respective groups were sacrificed and assessed for bacterial burden by determining the percentage of morulae-positive neutrophils using light microscopy and by PCR. Neutrophils were isolated from pooled splenocytes via positive selection using fluorescein isothiocyanate-conjugated rat anti-mouse Ly6G (Gr-1; a neutrophil-specific marker) MAb (BD Pharmingen, San Diego, CA) followed by anti-fluorescein isothiocyanate MAb conjugated to magnetic beads (Miltenyi Biotec, Auburn, CA). Total RNA was isolated from Gr-1-positive and -negative cells as described above and used as template for RT-PCR.

Statistical analyses. Statistical analyses were performed using the Prism 4.0 software package (Graphpad, San Diego, CA). If one-way analysis of variance (ANOVA) or two-way ANOVA indicated a group difference (P < 0.05), then Tukey’s or Bonferroni’s test was used, respectively, to test for a significant difference among groups. In some instances, the means for uninfected and infected samples per time point were examined using the Student t test.

Study approval. The Human Investigation Committees at Yale University and the University of Kentucky and the Yale University Institutional Animal Care and Use Committee approved these studies.

RESULTS

HL-60 cell fhc mRNA levels increase after a minimum of 24 h of A. phagocytophilum infection. We have previously reported that HL-60 and rHL-60 cells with established A. phagocytophilum infections exhibit increased fhc mRNA levels (14). However, neither the kinetics of fhc transcription immediately following A. phagocytophilum infection nor the effects of the bacterium on fhc expression are known. We therefore added host-cell-free A. phagocytophilum (~10 organisms per cell) to HL-60 cells, isolated total RNA, and performed quantitative RT-PCR (QRT-PCR) to assess changes in fhc and flc expression over 96 h of infection. For all samples, fhc mRNA levels exceeded those of flc by as much as 57.0- to 297.1-fold (Fig. 1). For the initial 12 h of infection, fhc mRNA levels of A. phagocytophilum-infected cells were 1.2- to 1.7-fold lower than those of uninfected control cells. Consistent with our previous study (14), however, fhc expression by A. phagocytophilum-infected cells increased significantly above those of uninfected controls by 24 h postinfection (hpi) and remained so throughout the duration of the time course. The largest rise in fhc expression, a 2.6-fold increase, was observed at 96 hpi. With the exception of 30 min postinfection, little to no change was observed in fhc transcription between infected and uninfected control HL-60 cells. These data demonstrate that the increased fhc mRNA levels associated with A. phagocytophilum infection of HL-60 cells are not observable until at least 24 hpi.

Ferritin protein levels in HL-60 cells decrease in a dose- and time-dependent manner following A. phagocytophilum infection. Though transcriptional expression does play a role in modulating ferritin levels within eukaryotic cells, ferritin homeostasis is primarily regulated at the level of protein translation (23, 24). We therefore investigated changes in ferritin content in HL-60 cells following A. phagocytophilum infection. Host-cell-free A. phagocytophilum organisms were added to HL-60 cells at approximate ratios of 0, 0.4, 1.1, 3.3, and 10 bacteria per cell. Four days postinfection, whole-cell lysates were extracted and analyzed. Samples exhibited no difference in actin levels upon immunoblot analysis, thereby demonstrating they were effectively normalized (Fig. 2A). As expected, a dose-dependent increase in A. phagocytophilum P44 was observed. When examined by ELISA, ferritin protein levels decreased in a dose-dependent manner in response to A. phagocytophilum burden (Fig. 2B). HL-60 cells that were inoculated with 10 organisms per cell demonstrated a threefold decline in ferritin levels at 96 hpi. This decline was maximal, as cultures that were inoculated with 30 bacteria per cell exhibited nearly identical decreases (data not shown). To assess the effect of A. phagocytophilum on HL-60 cell ferritin protein expression at earlier time points, host-cell-free bacteria were added at approximate ratios of 0, 1, and 10 per cell, and the time course was repeated. At 24, 48, and 96 hpi, whole-cell lysates were assessed for ferritin content. While ferritin levels increased per respective samples over the duration of the time course, A. phagocytophilum-infected cells exhibited a dose-dependent decrease in ferritin relative to uninfected controls (Fig. 2C).
Significant decreases in ferritin levels were apparent for cells inoculated with 10 organisms per cell as early as 24 hpi, which demonstrated a 1.7-fold reduction. A significant decrease in ferritin expression for cells inoculated with 1 bacterium per cell was not evident until 96 hpi. The reduction in ferritin levels in infected HL-60 cells relative to uninfected controls is apparent as early as 30 min postinfection and continues throughout the initial hours of *A. phagocytophilum* infection (Fig. 2D). However, the differences in ferritin levels between infected and uninfected controls are not statistically significant until 24 to 48 hpi. The observed changes in ferritin levels are not in response to fluctuations in whole-cell iron concentrations, which remained at levels that were too low to be detected by the ferrozine binding assay (43) throughout the time courses (data not shown). Thus, these results demonstrate that *A. phagocytophilum* infection of HL-60 cells results in a bacterial dose- and time-dependent reduction in host cell ferritin levels.

**Human neutrophil flc levels rapidly increase in response to *A. phagocytophilum* infection in vitro.** The effects of *A. phagocytophilum* on ferritin transcription in neutrophils are unknown. Furthermore, while the promyelocytic HL-60 cell line is permissive to *A. phagocytophilum*, it does not accurately mimic the natural mammalian host cell for the bacterium. For instance, HL-60 cells are devoid of specific granules and are unable to generate a robust respiratory burst compared to neutrophils. We and others have previously demonstrated that following binding to the neutrophil surface in vitro, *A. phagocytophilum* internalization is a prolonged process that lasts for several hours (8, 12, 25). This prolonged association of the bacterium with the neutrophil outer membrane stimulates at least some degree of NADPH oxidase assembly and degranulation (12). Because of ferritin’s role as a cytoprotectant against oxidative damage and to better understand the neutrophil expression profile in response to intracellular infection, we investigated changes in *fhc* and *flc* mRNA levels in neutrophils.

**FIG. 2.** Ferritin protein levels in HL-60 cells decrease in a time- and dose-dependent manner following *A. phagocytophilum* infection. Host-cell-free *A. phagocytophilum* (Ap) organisms were added to 5 × 10⁵ HL-60 cells at ratios of 0.4, 1.1, 3.3, and 10 per cell. Uninfected HL-60 cells served as controls. Four days postinfection, (A) whole-cell lysates (10 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western immunoblot analysis using antibodies directed against *A. phagocytophilum* P44 and actin, and (B) whole-cell lysates (30 μg) were assayed for ferritin content by ELISA. Bovine serum albumin (BSA; 30 μg) served as a negative control. Statistical significance was determined using one-way ANOVA followed by Tukey’s test. (C) Dose-dependent infection time course. *A. phagocytophilum* was added to 5 × 10⁵ HL-60 cells at approximate ratios of 0 (white bars), 1 (gray bars), and 10 (black bars) per cell. Whole-cell lysates (30 μg) were assessed for ferritin content at 24, 48, and 96 h postinfection. Statistical significance was determined using two-way ANOVA followed by Bonferroni’s test. The mean values indicated by different letters are significantly different. (D) Host-cell-free *A. phagocytophilum* organisms were added to HL-60 cells at a ratio of approximately 10 organisms per cell, and infection was allowed to proceed for 0, 0.25, 0.5, 1, 2, 5.5, 8, 12, 24, 48, 72, or 96 h. Uninfected HL-60 cells served as controls. Whole-cell lysates (30 μg) generated from 5 × 10⁶ cells per time point were assayed for ferritin content. The means ± standard errors of duplicate samples are presented. Statistical significance (*, P < 0.05; **, P < 0.01) was determined using two-way ANOVA followed by Bonferroni’s test. Results are representative of four to six independent experiments. Ctrl, control.
during the early hours of *A. phagocytophilum* infection. Human neutrophils were incubated in the presence and absence of host-cell-free *A. phagocytophilum* at an approximate ratio of 10 organisms per cell. The kinetics of infection paralleled previous observations (12) and achieved a maximum of 90% infection in HL-60 cells, neutrophils incubated with *A. phagocytophilum* demonstrated successive rises in not only *fhc* transcription but also *flc* transcription throughout the course of infection (Fig. 3A).

To further define the relevance of the observed changes in ferritin transcription associated with *A. phagocytophilum* infection, the time course was repeated with neutrophils being incubated with host-cell-free *A. phagocytophilum*, *Escherichia coli*, or OpZ. The latter two stimuli served as controls for activating neutrophil NADPH oxidase and degranulation. Changes in *fhc* and *flc* expression were quantified using qRT-PCR. Similar to that observed for HL-60 cells, *flc* levels exceeded *fhc* levels by as much as 20.6-fold (Fig. 3B). Consistent with the semiquantitative RT-PCR studies, *A. phagocytophilum* infection stimulated a twofold, statistically insignificant rise in *fhc* expression. Neutrophils incubated with *E. coli*, however, demonstrated as much as a 12.8-fold increase in *fhc* transcript levels. Incubation with OpZ yielded as much as a 3.2-fold rise in *fhc* message, though this change was not significant. In contrast to that observed for neutrophil *fhc* mRNA levels, *flc* transcription significantly increased following incubation with *A. phagocytophilum*, with increases of 16.2-, 20.0-, and 62.9-fold, respectively. Ferritin light-chain expression also significantly increased during the initial hours of *A. phagocytophilum* infection stimulated a twofold, statistically insignificant rise in *fhc* expression. Neutrophils incubated with *E. coli*, however, demonstrated as much as a 12.8-fold increase in *fhc* transcript levels. Incubation with OpZ yielded as much as a 3.2-fold rise in *fhc* message, though this change was not significant. In contrast to that observed for neutrophil *fhc* mRNA levels, *flc* transcription significantly increased following incubation with *A. phagocytophilum*, with increases of 16.2-, 20.0-, and 62.9-fold, respectively. Ferritin light-chain expression also significantly increased in response to *E. coli* and OpZ. Thus, ferritin expression, particularly that of *flc*, rapidly increases during the initial hours of *A. phagocytophilum* infection and upon exposure to phagocytic stimuli such as *E. coli* and OpZ.

**Neutrophil ferritin protein levels increase during the initial hours of *A. phagocytophilum* infection in vitro.** Neutrophil *fhc* transcription exhibits a low level of increase, while *flc* mRNA expression increases dramatically during the initial hours of *A. phagocytophilum* infection. Similar transcriptional profiles were also observed upon incubating neutrophils with *E. coli* and OpZ. We therefore examined whether the observed increases in ferritin transcription in neutrophils translates to rises in ferritin protein levels following incubation with each of these stimuli. Host-cell-free *A. phagocytophilum*, *E. coli*, or OpZ was added to neutrophils, after which whole-cell lysates were subjected to RT-PCR. Samples were normalized to *β*-actin and converted to cDNA, which was used as template for real-time PCR to quantify the relative expression levels of *fhc* and *flc*. Samples were analyzed in triplicate. Data are presented as the mean copies of either *fhc* or *flc* transcript per 10^6* β*-actin transcript copies. Error bars indicate standard deviations. Statistical significance was determined using one-way ANOVA followed by Tukey’s test. The mean values indicated by different letters are significantly different. Results are representative of four independent experiments.
were examined for ferritin content at 1, 3, and 6 hpi. At 1 hpi, neutrophil ferritin levels exhibited a three- to fourfold increase for all stimuli relative to uninfected controls (Fig. 4). Ferritin levels of A. phagocytophilum-infected cells remained high throughout the time course, while those of cells exposed to E. coli had declined to levels that were not significantly different from those of uninfected controls by 3 hpi. Ferritin expression by neutrophils exposed to OpZ had also dropped considerably at 3 and 6 hpi. Thus, exposure of neutrophils to A. phagocytophilum results in a sustained rise in ferritin levels, while exposure to either E. coli or OpZ stimulates an initially pronounced spike followed by a subsidence in ferritin amounts. The kinetics of ferritin expression induced by A. phagocytophilum, E. coli, and OpZ parallel the effects each of these stimuli has on NADPH oxidase assembly and degranulation in vitro (14). These data are consistent with the induction of ferritin expression as a cytoprotective response to ROS.

Changes in fhc and flc expression during murine A. phagocytophilum infection. To extend our analyses in vivo, C3H/HeJ mice were inoculated with A. phagocytophilum. Sham-inoculated mice served as controls. On days 2 and 8, the respective groups were sacrificed and Gr-1-positive and -negative cells were isolated. Total RNA was extracted and used as template for RT-PCR analyses targeting murine fhc and flc and A. phagocytophilum p44. Samples were normalized according to murine hypoxanthine phosphoribosyltransferase (HPRT) transcript levels. Transcript levels for fhc, flc, and the HPRT gene for Gr-1-negative cells on day 2 are similar to those observed for day 8 and thus are not shown. Results are representative for four separate experiments. ctrl, control.

There was no detectable difference in fhc expression between neutrophils from infected and uninfected mice on day 2. Notably, though, flc transcription was strongly up-regulated in neutrophils from infected mice on day 8, which typically corresponds to peak infection. These profiles are highly consistent with what would be expected to occur in the context of a strong IFN-γ response (47), which is a common manifestation of A. phagocytophilum infection (2, 20, 33). Thus, in vivo A. phagocytophilum infection results in a pronounced increase in neutrophil flc expression, a pattern that is consistent with the IFN-γ-dominated cytokine profile associated with infections by obligate intracellular pathogens.

DISCUSSION

A major function of ferritin is to limit Fe(II) available to participate in the generation of ROS. Oxidative stress activates transcriptional and posttranscriptional pathways of ferritin regulation (47). For instance, oxidants have been shown to increase fhc and flc expression in liver cell lines (48). Additionally, transcriptional induction of fhc and flc occurs in rat liver following injection with phorone, which inhibits free radical defense mechanisms by reducing glutathione concentration (11). Furthermore, eukaryotic cells enriched for ferritin H or ferritin L levels by either transfection (21, 36, 40) or pinocytosis (29) demonstrate a reduced LIP and, consequently, enhanced resistance to H2O2-induced oxidative damage. Conversely, cells in which flc expression is downregulated via an antisense oligonucleotide are more susceptible to oxidative insult (29).

As such, ferritin expression during the initial hours of A. phagocytophilum infection in vitro likely occurs as a response to ROS production during bacterial binding and invasion. Neutrophil transcription of ferritin heavy and light subunits is induced by as much as 12.8- and 62.9-fold, respectively, following exposure to A. phagocytophilum, E. coli, or OpZ, with a concomitant three- to fourfold rise in ferritin protein levels. The elevated protein levels in response to A. phagocytophilum are maintained throughout the course of infection, while those in response to E. coli and OpZ subside. The initial spike and subsidence of ferritin expression in response to E. coli and OpZ are consistent with the kinetics of ROS production associated with phagocytosis of each of these stimuli (12). Similarly, the maintained elevation in neutrophil ferritin content throughout the initial hours of A. phagocytophilum infection is consistent with a sustained, low-level stimulation of NADPH oxidase. We have previously demonstrated that internalization of A. phagocytophilum in vitro occurs over several hours following bacterial binding to neutrophil surfaces and that this process is associated with some degree of host cell activation, NADPH oxidase mobilization, and degranulation (12). Furthermore, A. phagocytophilum infection also stimulates neutrophil transcription of superoxide dismutase, which further evidences the induction of a cytoprotective response (8, 38).

The rises in ferritin content observed for neutrophils upon exposure to A. phagocytophilum were not noted for HL-60 cells. This is likely because promyelocytic HL-60 cells lack specific granules and express low levels of NADPH oxidase components compared to mature neutrophils (1, 7, 46). Consequently, stimulation of HL-60 cells elicits O2− at levels that are orders of magnitude lower than those observed upon
neutrophil stimulation. As such, the addition of *A. phagocytophilum* to HL-60 cells yields little ROS production relative to that observed for neutrophils (5, 12).

The induction of *fhc* expression in neutrophils over 6 h of *A. phagocytophilum* infection in vitro is minor compared to the considerable increase in *fhc* message observed on day 8 of in vivo infection. It is well established that IFN-γ plays a major role in host defense against intracellular pathogens. Accordingly, IFN-γ dominates the host cytokine response to *A. phagocytophilum* infection, with the highest levels being produced between days 5 and 10 (2, 20). It has been demonstrated that incubation of THP-1 cells in the presence of IFN-γ strongly up-regulates *fhc* mRNA expression with a concomitant increase in ferritin protein (44). Given the influence of IFN-γ on *fhc* expression, it is not surprising that the ferritin transcription profile of the entire neutrophil population of *A. phagocytophilum*-infected mice at the peak of bacteremia displays a pronounced shift from *fhc* to *fhc*.

HL-60 cells demonstrate a marked increase in *fhc* mRNA synthesis during the course of *A. phagocytophilum* infection, as confirmed by cDNA microarray screening (14) as well as semi-quantitative and QRT-PCR. A similar result was noted for THP-1 cells infected with *Mycobacterium bovis* BCG (28). Thus, an increase in *fhc* expression may exemplify a common theme among host expression profiles associated with intracellular bacterial infection.

Current knowledge supports that, with the exception of *Borrelia burgdorferi* (41), all pathogenic bacteria require iron as a metabolic cofactor (37). The mechanism by which *A. phagocytophilum* obtains iron from its host cell or whether acquiring iron is necessary for this bacterium’s survival is unknown. Barnewall and colleagues demonstrated that *Ehrlichia chaffeensis* and *E. senetts*, but not *A. phagocytophilum*, exploit the transferrin receptor pathway as a means for obtaining iron that is brought into the host cell before it can be utilized for host metabolic processes or stored by ferritin (6, 34). Within 30 min of *A. phagocytophilum* infection, HL-60 cell ferritin protein levels are lowered relative to uninfected controls, though the reduction is not statistically significant until at least 24 hpi. Given the slow doubling time of *A. phagocytophilum* (9), it is not surprising that the decline in ferritin content relative to uninfected cells does not become significant until this time point, as this effect is bacterial dose dependent. Ferritin light-subunit mRNA expression exhibits little to no change for infected HL-60 cells at all time points, while *fhc* mRNA levels are significantly elevated at 24, 48, and 96 hpi. Thus, the loss of ferritin protein is not a consequence of transcriptional inhibition. This suggests the possibility that *A. phagocytophilum* may either degrade or promote the degradation of ferritin protein, which would release iron for use by the bacterium. Support for this speculation is offered by the recent report by Larson and colleagues that *Neisseria meningitidis* accelerates degradation of ferritin within A431 epithelial cells to yield a useable iron source (27). As observed for *A. phagocytophilum*-infected HL-60 cells, *fhc* and *fhc* mRNA levels of *N. meningitidis*-infected epithelial cells remain relatively unchanged while ferritin protein levels are significantly reduced over the course of infection. This may represent a common theme among intracellular pathogens, as *Legionella pneumophila* (10, 22), *Mycobacterium tuberculosis* (35), and *Listeria monocytogenes* (45) have also been shown to access intracellular iron stores by undefined mechanisms.

While a mechanism for Fe(III) release via host cell ferritin degradation has been documented for *N. meningitidis*, a corresponding neisserial iron binding protein has yet to be identified. Doyle and colleagues recently identified a ferric ion-binding protein (FBP) homolog in *Ehrlichia canis* and *E. chaffeensis* (18). Putting this information into context with data presented in this report and by Larson et al. (27), it is tempting to surmise whether *A. phagocytophilum* also expresses an FBP homolog and whether it plays a role in acquiring Fe(III) released from degraded ferritin.

These studies provide insight into the effects *A. phagocytophilum* has on the ferritin levels of its mammalian host at different stages of infection. The potential role of ferritin degradation by *A. phagocytophilum* as a means for obtaining iron and whether an *Ehrlichia*-like FBP is involved in this process represent exciting areas for further investigation.

**ACKNOWLEDGMENTS**

We thank Ralph Horowitz of New York Medical College and Yasuko Rikihisa of Ohio State University for providing *A. phagocytophilum* strain HZ and Jennifer Marcum for technical assistance.

This work was supported by grants KO1DK065039 (J.A.C.), P20 RR20171 (J.A.C.), and AI041440 (E.F.) from the National Institutes of Health and the Burroughs Wellcome Fund (E.F.).

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