Coxiella burnetii Infection Increases Transferrin Receptors on J774A.1 Cells†

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Inoculation with viable, but not inactivated, Coxiella burnetii resulted in the increased expression of transferrin receptors (TfR) in the murine macrophage-like cell line J774A.1. This upregulation was evident in immunoblots as early as 6 h postinfection, with TfR levels continuing to increase through the first 24 h of infection. Fluorescent labeling revealed that TfR upregulation occurred throughout infected monolayers, eliminating the possibility that it reflected a response by a minor subset of host cells. In addition, TfR trafficking did not appear to be affected by C. burnetii infection. Consistent with the increase in TfRs, inoculation with viable C. burnetii resulted in a 2.5-fold increase in total cellular iron by 12 h postinoculation. Our further findings that the chelation of intracellular iron arrests C. burnetii replication and that C. burnetii metabolic activities in vitro are affected by iron concentration suggest that TfR upregulation is a salient factor in C. burnetii infection, and we speculate that it may represent a significant virulence mechanism.

Intracellular pathogens obtain shelter from the immune response and access to intracellular nutrients from within their hosts. However, to exploit this niche, these parasites must be able to evade the innate destructive mechanisms of their host cells. Organisms such as Mycobacteria tuberculosis (17), Chlamydia psittaci (14, 55), and Legionella pneumophila (26) achieve this evasion by preventing phagolysosomal fusion, while Shigellae (20, 49) and Listeria monocytogenes (18) escape from endosomes into the cytoplasm. Both mechanisms allow these bacteria to survive by avoiding the low pH, oxygen radicals, and degradative proteases of the phagolysosome.

Coxiella burnetii, however, employs no mechanisms to avoid the phagolysosome and indeed requires this harsh environment for replication. Vacuoles containing C. burnetii have characteristics of typical phagolysosomes, as shown by the presence of lysosomal membrane markers in vacuole membranes (23) as well as staining positively for the lysosomal enzymes, 5′-nucleotidase (8), and acid phosphatase (1, 8). The internal pH of vacuoles containing C. burnetii, about 4.8 (35), is identical to that of phagolysosomes. This acidic pH appears to be essential for C. burnetii metabolic activity. In vitro experiments find that C. burnetii is able to catabolize glucose and glutamate, generate ATP, and synthesize nucleic acids and proteins at low but not neutral pH. This is consistent with the in vivo observation that C. burnetii replication is inhibited by raising the phagolysosomal pH with lysomotrophic amines (26), however, employs no mechanisms to avoid the phagolysosome and indeed requires this harsh environment for replication. Vacuoles containing C. burnetii have characteristics of typical phagolysosomes, as shown by the presence of lysosomal membrane markers in vacuole membranes (23) as well as staining positively for the lysosomal enzymes, 5′-nucleotidase (8), and acid phosphatase (1, 8). The internal pH of vacuoles containing C. burnetii, about 4.8 (35), is identical to that of phagolysosomes. This acidic pH appears to be essential for C. burnetii metabolic activity. In vitro experiments find that C. burnetii is able to catabolize glucose and glutamate, generate ATP, and synthesize nucleic acids and proteins at low but not neutral pH. This is consistent with the in vivo observation that C. burnetii replication is inhibited by raising the phagolysosomal pH with lysomotrophic amines (26).

In contrast, C. burnetii is adapted to a phagolysosomal environment where it is able to flourish by means that are as yet poorly understood. Current evidence indicates that upon infection C. burnetii is able to modify responses that accompany phagocytosis, perhaps ameliorating its environment to some degree. C. burnetii infection is known to reduce the respiratory burst of macrophages, an integral part of the bactericidal response during infection (3). Phagolysosomes containing C. burnetii appear to be more fusion permissive than phagolysosomes inhabited by another obligate intracellular pathogen, Leishmania amazonzensis (42, 52, 53). Additionally, phagolysosomes containing C. burnetii coalesce into a single large vacuole, indicating fusion permissibility (22).

The present study documents another consequence of C. burnetii infection, an increase in transferrin receptor (TfR) expression. The significance of these observations is twofold. First, we find that intracellular iron is essential for C. burnetii replication, and thus upregulation of TfR may facilitate C. burnetii sequestration of this vital nutrient. Second, an increase in the intracellular iron in macrophages is known to depress killing of intracellular pathogens (37), and thus upregulation of TfR may promote C. burnetii survival.

MATERIALS AND METHODS

Host cell lines and bacterial propagation. J774A.1, a murine macrophage-like cell line (TIB-67; American Type Culture Collection, Rockville, Md.), was maintained in RPMI 1640 medium (RPMI; Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 5% CO2 at 37°C. Coxiella burnetii Nine Mile phase I (48) was propagated in J774A.1 cells in the same conditions as noted above. C. burnetii was harvested by differential centrifugation of cell culture supernatants first at 5,500 × g for 10 min to pellet host cell debris and then at 15,000 × g for 1 h to pellet the bacteria. Numbers of C. burnetii were estimated by measuring the turbidity of bacterial suspensions with a Klett Summerson photoelectric colorimeter (Klett Manufacturing Company Incorporated, New York, N.Y.), using a no. 42 filter (40); for a given experiment, densities were adjusted to provide the desired multiplicity of infection (MOI). C. burnetii was inactivated either chemically, by incubation for 12 h at room temperature with vigorous shaking in 2% glutaraldehyde–2% paraformaldehyde, or physically, by incubation for 2 h at 80°C. C. burnetii inactivated by both these methods were compared in all experiments, and no differences were seen (results not shown).

Immunoblotting. J774A.1 cells were seeded onto six-well Falcon plates (VWR Scientific Products, Seattle, Wash.) at 104 cells per well, allowed to adhere for 12 h, and then incubated for 1 h with viable or inactivated C. burnetii at 105 per ml in RPMI–10% FBS. Extracellular C. burnetii was removed by three washes with medium at 37°C. At designated time points, the medium was removed, cells were washed three times with phosphate-buffered saline (PBS; 0.1 M NaPO4, 0.1 M NaCl [pH 7.4]), and cell numbers were determined. Cells were pelleted, suspended to 5 × 106 per ml in deionized distilled water containing 2 mM phenylmethylsulfonyl fluoride, and frozen. Samples of 5 × 106 cells were denatured by boiling in Laemmli’s buffer (30), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels, then electrotransferred to polyvinylidene difluoride membranes by standard techniques. Membranes were blocked in Blotto (PBS with 10% skim milk powder and 0.1% Tween 20) for 1 h followed by protein transfer and then probed with rat anti-mouse TfR monoclonal antibodies (kindly provided by R. Hyman, Salk Institute, San Diego, Calif.) (32) followed by goat anti-rat immunoglobulin G conjugated to horseradish peroxidase (Pierce, Rockford, Ill.). Signal was visualized with an enhanced chemiluminescence kit (Amersham, Cleveland, Ohio).

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† This paper is dedicated to the memory of Louis P. Mallavia.
and images were analyzed with an Image Master scanning densitometer and associated software (Pharmacia Biotech, Uppsala, Sweden).

**Fluorescent labeling of TfR.** Confocal microscopy was used to compare TfR expression seen during *C. burnetii* infection with that seen in response to soluble factors in conditioned medium. J774A.1 cells were seeded on 15-mm-diameter Thermolon cover slips (Fisher Scientific, Santa Barbara, Calif.) in 24-well plates at 2.5 × 10⁶ per well and allowed to adhere for 12 h. Then they were inoculated with viable or inactivated *C. burnetii* (both in RPMI-10% FBS) or exposed to filter-sterilized conditioned medium (harvested from a 3-day culture of persistently infected J774A.1 cells) for an additional 16 h. At the end of this period, more than 95% of the cells were found to have taken up the inoculum. Following treatment, coverslips were rinsed with PBS, fixed in 0.1 M Pierce HCl–2% paraformaldehyde–7.25 mM sodium periodate in 0.1 M NaPO₄ buffer (pH 7.4) for 1 h, and washed three times in PBS. The samples were then blocked and permeabilized with 0.1% gelatin–0.3% saponin in PBS for 15 min, incubated with rat anti-mouse TfR monoclonal antibody for 1 h, washed three times, and probed with a fluorescein isothiocyanate-labeled rat secondary antibody (Organon Teknika Corp., West Chester, Pa.) for 1 h. Secondary antibody was washed off once for 10 min with block solution and twice for 10 min with PBS. Samples were fixed for 1 h at room temperature in PBS containing 4% paraformaldehyde.

A Prolong antifade kit (Molecular Probes Inc., Eugene, Oreg.) was used as a mounting medium. Micrographs were obtained with a Nikon inverted microscope equipped with a krypton-argon laser (Bio-Rad) on a Nikon inverted microscope equipped with an A Florescein isothiocyanate-labeled rat secondary antibody (Organon Teknika Corp., West Chester, Pa.) for 1 h. Secondary antibody was washed off once for 10 min with block solution and twice for 10 min with PBS. Samples were fixed for 1 h at room temperature in PBS containing 4% paraformaldehyde.

**Determination of intracellular iron content.** J774A.1 cells were seeded onto six-well plates at 10⁵ cells per well, allowed to adhere for 12 h, and then incubated for 1 h with viable or inactivated *C. burnetii* at 10⁶ per ml in RPMI-10% FBS. Removal of extracellular *C. burnetii* was facilitated by three washes with 37°C medium at designated time points, the medium was removed, cells were washed three times with 0.7% saline solution, and cells were removed by scraping. Following determination of cell numbers and viability, cells were pelleted, washed, and resuspended in 37°C medium at designated time points. For analysis, samples were suspended in 100 μl of a 6 N HClO₄, 5 × 10⁵ cells per ml, incubated at room temperature for 12 h, heated to 90°C for 3 h, and then diluted to 1 ml with deionized distilled H₂O. Iron content was then determined by inductively coupled plasma emission, using a Jobin Yvon JY24 instrument (Instrumental Services, Livermore, Calif.).

**Chelation of intracellular free iron.** J774A.1 cells were seeded at 4 × 10⁵ per well onto six-well plates and allowed to adhere for 12 h. Cells were incubated for 24 h with *C. burnetii* resuspended at 10⁵ per ml in RPMI-10% FBS containing fresh 0, 20, or 100 μM deferoxamine (DNX; Sigma, St. Louis, Mo.). Extracellular *C. burnetii* was removed by washing, and incubations continued in medium containing DNX. Cultures were evaluated at 24, 48, and 72 h postinfection. Cells were stained with the viability indicator trypan blue, and total cell numbers were determined by hemocytometer. Time course studies revealed that upregulation of TfR expression in infected J774A.1 cells began between 3 and 6 h postinfection, with the greatest increase evident between 9 and 12 h, and continued to increase at least 24 h postinoculation, at which time the optical density of TfR bands was approximately 4.5-fold greater than that in the uninfected control cells (Fig. 2). There was no apparent increase in TfR expression in cells inoculated with inactivated *C. burnetii*, suggesting that live organisms were required for TfR upregulation.

**RESULTS**

**TIR expression in macrophages during infection by *C. burnetii*.** Intracellular trafficking of iron in mammalian systems utilizes the iron carrier protein transferrin. Certain extracellular parasites such as Neisseria and Haemophilus spp. exploit this system by producing their own receptors for transferrin, through which they harvest sufficient iron to meet their metabolic needs (11). More recently, the intracellular parasite *Ehrlichia chaffeensis* was found to inhabit an endosome that accumulates TIRs in conjunction with an increase in TIR mRNA (5). In the present study, immunoblot analysis of SDS-PAGE-resolved lysates from uninfected and *C. burnetii*-infected cells were examined by using a monoclonal antibody specific for the murine TfR to determine if *C. burnetii* similarly modified TfR expression.

J774A.1 cells were inoculated with viable *C. burnetii* at MOIs ranging from 10 to 2,000 organisms per cell and then incubated for 12 h prior to sampling. Cells inoculated at an MOI of 100 or greater displayed an increase in TfR receptor expression. Time course studies revealed that upregulation of TfR expression in infected J774A.1 cells began between 3 and 6 h postinfection, with the greatest increase evident between 9 and 12 h, and continued to increase at least 24 h postinoculation, at which time the optical density of TfR bands was approximately 4.5-fold greater than that in the uninfected controls (Fig. 2). There was no apparent increase in TfR expression in cells inoculated with inactivated *C. burnetii*, suggesting that live organisms were required for TfR upregulation.

**TIR distribution and subcellular location.** Confocal microscopy was used to visualize the increase in TIR after *C. burnetii* infection (Fig. 3). The intensity of fluorescence in cells infected with *C. burnetii* was uniformly distributed and 1.5-fold greater than that seen in either cells treated with inactivated *C. burnetii* or uninoculated control cells. Both infected cells and cells treated with conditioned medium exhibited a morphological change from spread out to rounded up (Fig. 3A and 3B). However, this morphological change did not correlate with increased TIR expression, as there was no increase in the fluorescence of cells incubated in conditioned medium, demonstrating that soluble factors were not responsible for TIR upregulation. The distribution of fluorescent label on the outside of *C. burnetii*-infected J774A.1 cells showed no subcellular examination by transmission electron microscopy of TIR dis-
TfR expression and total intracellular iron following infection suggests that C. burnetii is able to obtain iron from the intracellular pool of free iron.

**DISCUSSION**

In living organisms, the intracellular iron concentration must be tightly regulated. Iron is essential for a variety of cellular processes, including electron transport, numerous redox reactions, and DNA biosynthesis. Iron is also toxic, catalyzing the generation of oxygen radicals via the Haber reaction, damaging DNA and cellular membranes (2, 36, 47). Iron uptake in mammalian cells is controlled by the expression of TfRs and the concentration of extracellular iron-loaded transferrin. TfRs are upregulated in response to the depletion of intracellular iron (24, 54). This regulation occurs at the posttranscriptional level by stabilization of TfR mRNA through the interaction of iron-responsive proteins with iron-responsive elements on mRNA (41). There appear to be additional uncharacterized pathways, as exemplified by cytokine-induced control of TfR expression (54). In this study, an increase in TfR expression was observed when J774A.1 cells were infected with viable C. burnetii. Mechanisms that elicit this response are unknown. Soluble factors released by infected cells did not appear to be responsible for TfR upregulation. It is possible that C. burnetii interacts directly with regulation pathways that lead to TfR upregulation. On the other hand, host cells may respond to a decrease in available iron as a result of iron sequestration by C. burnetii.

To move iron into a mammalian cell, TfR binds transferrin, an iron binding serum protein, at the plasma membrane of the cell. The TfR-transferrin complex is internalized via a clathrin-coated pit, which is then incorporated into an early endosome (7, 21). Here vacuolar ATPas lowers the internal pH of the endosome to about 5.5 (50), and in response to the lowered pH, the transferrin releases the iron while remaining bound to the TfR (33, 39). The empty transferrin (or apotransferrin)-TfR complex is returned to the plasma membrane; here the
neutral pH causes the TfR to lose its affinity for the apotransferrin, and it is released into the serum (12, 28). Thus, TfRs are cycled from the plasma membrane to endosomes (25) and back to the plasma membrane constitutively carrying iron into the cell as long as iron-loaded transferrin is available.

The TfRs upregulated during C. burnetii infection appear to cycle normally, as demonstrated by the fluorescent labeling seen in Fig. 3, and one would expect to see an increase in intracellular iron levels. We found a doubling of intracellular iron by 12 h postinfection, but by 24 h it returned to the preinfection level. Because TfR levels do not return to preinfection levels at this same rate, we speculate that homeostatic mechanisms maintaining intracellular iron balance may achieve this balance (43). Establishment of increased iron traffic may provide an iron source for replicating C. burnetii.

In addition to enhancing the availability of an essential nutrient, the increase in intracellular iron associated with C. burnetii upregulation of TfRs may allow the proliferation of these intracellular pathogens within macrophages. A diet rich in iron enhances infection by intracellular pathogens, while an iron-deficient diet reduces infection (31, 37, 46). In vitro studies have shown that increased intracellular iron levels allow the proliferation of intracellular pathogens (13, 15, 34, 44), some by reversing the gamma interferon stimulation of macrophages that results in the killing of intracellular pathogens (4, 9, 29). The mechanisms of killing by gamma interferon-activated macrophages include an enhancement of the oxidative burst (38), an increase in nitric oxide levels (27), and the downregulation of TfRs accompanied by a reduction in intracellular iron (6, 9, 51). In macrophages infected with C. burnetii, the oxidative burst is reduced (1) and TfR levels are upregulated as reported here, possibly diminishing the ability of macrophages to eliminate these intracellular pathogens.

Certain intracellular pathogens appear to access free iron pools and indeed may require this free iron pool (5, 45). DfX removes intracellular free iron or iron which is not bound by host-chelating proteins. Treatment of infected mammalian cells with DfX halts proliferation of intracellular Plasmodium...
were mounted on slides, and harvested at 24 h, and six wells were harvested at 72 h. The number and viability of J774A.1 cells per well were determined by hemocytometer. Infected J774A.1 cells by inductively coupled plasma emission of J774A.1 cell cultures inoculated with

Error bars for two points at 36 h, as one data point was available for cells inoculated with inactivated C. burnetii and two were available for uninoculated controls.


tables were done in triplicate, and error bars indicate standard deviation. There are no uninoculated control (triangles). The results shown are the means for an experiment done in triplicate.


J774A.1 cell by J774A.1 cells per well for each well harvested. Viability of J774A.1 cells remained above 80% for all samples. Results are means ± standard deviations.


**FIG. 4.** An increase in intracellular iron would be expected to result from an upregulation of TIRs. The graph shows the intracellular iron content determined by inductively coupled plasma emission of J774A.1 cell cultures inoculated with viable (open circles) and inactivated C. burnetii (filled circles) as well as an uninoculated control (triangles). The results shown are the means for an experiment done in triplicate, and error bars indicate standard deviation. There are no uninoculated control (triangles). The results shown are the means for an experiment done in triplicate, and error bars indicate standard deviation. There are no

**TABLE 1. Chelation of intracellular free iron with DfX halts C. burnetii proliferation**

<table>
<thead>
<tr>
<th>Cells</th>
<th>J774A.1 (10^6)/well</th>
<th>C. b./J774A.1 cell</th>
<th>C. b. (10^6)/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>24 h</td>
<td>24 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Infected 0 μM DfX</td>
<td>3.3 ± 1.1</td>
<td>14.0 ± 3.0</td>
<td>10.4 ± 2.5</td>
</tr>
<tr>
<td>20 μM DfX</td>
<td>4.9 ± 2.6</td>
<td>12.0 ± 1.9</td>
<td>9.5 ± 1.7</td>
</tr>
<tr>
<td>100 μM DfX</td>
<td>4.2 ± 0.2</td>
<td>6.9 ± 0.7</td>
<td>14.5 ± 0.5</td>
</tr>
<tr>
<td>Uninfected 0 μM DfX</td>
<td>4.5 ± 0.6</td>
<td>15 ± 0.6</td>
<td>NA</td>
</tr>
<tr>
<td>20 μM DfX</td>
<td>5.5 ± 1.3</td>
<td>11 ± 2.2</td>
<td>NA</td>
</tr>
<tr>
<td>100 μM DfX</td>
<td>4.8 ± 0.9</td>
<td>6.3 ± 1.0</td>
<td>NA</td>
</tr>
</tbody>
</table>

* J774A.1 cells in 24-well plates were infected with C. burnetii (C. b.) and simultaneously incubated in 0, 20, or 100 μM DfX. Three wells for each treatment were harvested at 24 h, and six wells were harvested at 72 h. The number and viability of J774A.1 cells per well were determined by hemocytometer. Infected J774A.1 cells were mounted on slides, and C. burnetii was visualized by Gimenez stain and counted on each slide. C. burnetii per well was calculated by multiplying C. burnetii per J774A.1 cell by J774A.1 cells per well for each well harvested. Viability of J774A.1 cells remained above 80% for all samples. Results are means ± standard deviations. NA, not applicable.

**FIG. 5.** To determine the effect of soluble iron on metabolic activity, C. burnetii was labeled with [35S]methionine for 2 h in a citrate buffer (pH 4.5) containing 0, 0.1, or 0.2 mM iron. Proteins expressed during this in vitro incubation were visualized by autoradiography to compare expression. Equal numbers of cells were loaded onto all lanes. Total expression is reduced by 38% in an average of five experiments at 0 mM iron, while expression of a 20-kDa and a 17-kDa protein (indicated by arrows) is reduced at 0.2 mM iron.

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* E. chaffeensis, L. pneumophila, and two were available for uninoculated controls.
REFERENCES


