INFECTION AND IMMUNITY, Apr. 1997, p. 1455–1461 Vol. 65, No. 4
0019-9567/97/$04.00+0

Ehrlichia chaffeensis Inclusions Are Early Endosomes Which Selectively Accumulate Transferrin Receptor

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Received 10 October 1996/Returned for modification 22 November 1996/Accepted 27 January 1997

Ehrlichia chaffeensis* is an obligatory intracellular bacterium which infects macrophages and monocytes. Double immunofluorescence labeling was used to characterize the nature of *E. chaffeensis* inclusion in the human promyelocytic leukemia cell line THP-1. *E. chaffeensis* was labeled with dog anti-*E. chaffeensis* serum and fluorescein isothiocyanate-conjugated anti-dog immunoglobulin G (IgG). Lissamine rhodamine-conjugated anti-mouse IgG was used to label various mouse monoclonal antibodies. Ehrlichial inclusions did not fuse with lysosomes, since they were not labeled with anti-CD63 or anti-LAMP-1. The ehrlichial inclusions were slightly acidic, since they weakly accumulated 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine and stained weakly positive for vacuolar type H^+ ATPase. Some ehrlichial inclusions were labeled positive with antibodies against HLA-DR, HLA-ABC, and β_2 microglobulin, while other inclusions in the same cell were labeled negative. The inclusions were labeled strongly positive for transferrin receptors (TfRs) and negative for the clathrin heavy chain. Time course labeling for TfRs showed that up to 3 h postinfection, most of the ehrlichial inclusions were negative for TfRs. After 6 h postinfection, 100% of the ehrlichial inclusions became TfR positive and the intensity of labeling was increased during the subsequent 3 days. Reverse transcription-PCR showed a gradual increase in the level of TfR mRNA postinfection, which reached a peak at 24 h postinfection. These results suggest that ehrlichial inclusions are early endosomes which selectively accumulate TfRs and that the ehrlichiae up-regulate TfR mRNA expression.

Ehrlichia chaffeensis, which belongs to the family *Rickettsiaceae*, is an obligatory intracellular bacterium of monocytes/macrophages (27). *E. chaffeensis* was first isolated in 1990 at Fort Chaffee, Ark., from a patient with human monocytic ehrlichiosis (9), which was first reported in the United States in 1987 (19). Since then, over 400 cases of human ehrlichiosis have been reported in 30 states. Serologic evidence suggests the presence of human monocytic ehrlichiosis in Europe (Spain [12], Portugal [22], and Belgium [26]) and Africa (30). Clinical signs include fever, headache, myalgia, arthralgia, nausea, vomiting, anorexia, chills, and a rash in some patients (10, 11). Elevations in levels of hepatic aminotransferases in sera and thrombocytopenia also occur. The severity of the disease can range from asymptomatic infection to severe morbidity and death in some instances (10, 11).

All ehrlichial species replicate and survive in host cell membrane-bound inclusions. However, what membrane-bound compartment ehrlichiae occupy is unknown. We have shown that *Ehrlichia risticii* selectively prevents lysosomal fusion with ehrlichia-containing inclusions (32). Recently, the intracellular bacteria *Mycobacterium tuberculosis* (7), *Mycobacterium avium* (29), *Legionella pneumophila* (7, 8), *Coxiella burnettii* (14), and *Chlamydia trachomatis* (14) were shown to occupy unique cytoplasmic membrane-bound compartments which are distinct from endosomes or phagolysosomes, and these compartments appear to be tailored for each microorganism.

The extreme sensitivity of ehrlichiae to the intracytoplasmic iron chelator deferoxamine (3, 24) suggests that iron is essential for ehrlichiae and that ehrlichiae do not possess iron-binding molecules with affinities higher than that of deferoxamine. How intracellular bacteria, such as ehrlichiae, acquire iron is unknown. The elucidation of how ehrlichiae acquire iron would further our understanding of ehrlichial survival in an antimicrobial effector cell.

This paper examines in what type of host membrane-bound inclusion *E. chaffeensis* resides by examining localization of various host cell membrane proteins characteristic of compartments in the endosome-lysosome pathway and attempts to determine if these proteins colocalize with ehrlichial inclusions. Furthermore, this paper examines whether ehrlichiae modulate a major host iron uptake mechanism, the human transferrin-human transferrin receptor (Tf-TfR) recycling pathway of the host cells.

MATERIALS AND METHODS

***E. chaffeensis* culture.** The Arkansas *E. chaffeensis* isolate was originally obtained from J. Dawson (Centers for Disease Control and Prevention, Atlanta, Ga.). *E. chaffeensis* was propagated in THP-1 cells (a human promyelocytic leukemia cell line kindly provided by M. D. Wewers, The Ohio State University, Columbus) incubated in RPMI 1640 medium (GIBCO-BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Atlanta Biological, Atlanta, Ga.) and 4 mM L-glutamine (GIBCO-BRL) at 37°C in 5% CO_2–95% air without antibiotics.

Preparation of host-cell-free *E. chaffeensis*. When more than 90% of the cells were infected, as determined by examination of cytocentrifuged (Cytospin 2; Shandon, Inc., Pittsburgh, Pa.) cells stained with Diff-Quik (Baxter Scientific Products, Obetz, Ohio), the infected cells were suspended in 5 ml of culture medium at 10^6 cells per ml, sonicated at a setting of 2 at 20 kHz for 7 to 8 s with an ultrasonic processor (model W-380; Heat Systems, Farmingdale, N.Y.), and then centrifuged at 500 × *g* for 5 min. The supernatant, which contained the host-cell-free ehrlichiae, was centrifuged at 10,000 × *g* for 10 min, and the pellet was used to infect THP-1 cells.

Double immunofluorescence labeling. THP-1 cells, 3 days postinfection, were fixed for 1 h with Nakane's fixative (21), which consists of 1% paraformaldehyde, 50 mM phosphate buffer (pH 7.4), 10 mM sodium periodate, and 75 mM lysine. The cells were permeabilized with buffer A, which consisted of phosphate-buffered saline (0.17 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4 [pH 7.4]), 0.1% gelatin, and 0.3% saponin. The cells were then labeled with primary mouse monoclonal antibodies in buffer A for 1 h at room temperature and washed three times with buffer A. Antibodies used were anti-human lyso-

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some-associated membrane protein 1 (LAMP-1, antibody H4A3) at a 1:10 dilution of the original supernatant (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), anti-human CD63 at a 1:10 dilution of the original solution (Immunotech, Inc., Westbrook, Maine), anti-57-kDa B subunit vacuolar type H^+ ATPase of the bovine chromaffin granule at a 1:50 dilution of the original solution (kindly provided by N. Nelson from the Roche Institute of Molecular Biology, Nutley, N.J.), anti-73-kDa subunit of vacuolar type H^+ ATPase of bovine-brain-coated vesicles at a 1:20 dilution of the original stock solution (kindly provided by M. Forgac, Tufts University), anti-TfR (Immunotech) at a 1:5 dilution of the original solution, anti-human HLA-DR (major histocompatibility complex [MHC] class II molecules) (Immunotech) at 6 μg/ml, anti-human HLA-ABC (which recognizes the MHC class I heavy chain associated with β_2 microglobulin) (Immunotech) at 6 μg/ml, anti-human β_2 microglobulin (Sigma, St. Louis, Mo.) at a 1:250 dilution of stock ascites fluid, and anti-human clathrin heavy chain (kindly provided by F. M. Brodsky, University of California, San Francisco) at 10 μg/ml. Lissamine rhodamine-conjugated anti-mouse immunoglobulin G (IgG) at 30 μg/ml (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was used to label the mouse monoclonal antibodies. The cells were then incubated with dog anti-*E. chaffeensis* serum at a 1:100 dilution and fluorescein isothiocyanate (FITC)-conjugated anti-dog IgG (Jackson) at 7.5 μg/ml. The dog anti-*E. chaffeensis* serum was developed in our laboratory by multiple inoculations of 5×10^7 to 8×10^7 highly infected DH82 cells into an adult, specific-pathogen-free, male dog. Prior to the first inoculation, serum was collected as a baseline control. After confirmation of the development of *E. chaffeensis* antibody by an indirect fluorescent antibody test (titer, 1:5,120), the blood was collected. The dog anti-*E. chaffeensis* serum had been preabsorbed with uninfected THP-1 cells at 10^6 cells/ml of serum at 37°C for 1 h. Negative controls consisted of uninfected THP-1 cells incubated with dog anti-*E. chaffeensis* serum and FITC-conjugated anti-dog IgG and infected THP-1 cells with secondary conjugated antibodies alone or with preimmune dog serum and FITC-conjugated anti-dog IgG. Cytocentrifuged preparations of the labeled cells were then mounted with a semipermanent mounting medium consisting of 2.4 g of polyvinyl alcohol (Mowiol 4-88; Calbiochem, La Jolla, Calif.) and 6.0 g of glycerol mixture in 6 ml of distilled water and 12 ml of 0.2 M Tris-HCl, pH 8.5. The cells were then viewed by epifluorescence microscopy.

DAMP labeling. Cells were labeled with 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP) with an acidic granule kit (Oxford Biomedical Research, Inc., Oxford, Mich.). Briefly, 2 ml of 10^7 infected or uninfected THP-1 cells/ml was incubated with 20 μl of DAMP solution at 37°C for 30 min. Cells were then fixed with Nakane's fixative and permeabilized as described previously. The cells were incubated with mouse anti-dinitrophenol (anti-DNP) at a dilution of 1:10 for 1 h at 37°C, washed with buffer A, and labeled with lissamine rhodamine-conjugated anti-mouse IgG. The cells were labeled for *E. chaffeensis* as described above and viewed by epifluorescence microscopy.

TfR and ehrlichia colocalization time course. Infected THP-1 cells (10^6) were fixed and double immunofluorescence labeled as described above with anti-TfR IgG and *E. chaffeensis* antibody at 0, 3, 6, 12, 24, 48, or 72 h postinfection.

FITC-Tf uptake study. After 2 days of infection, the fetal bovine serum concentration was reduced to 2% and FITC-holotransferrin (holoTf) (Immunotech) at 4 μg/ml was added to the THP-1 cells. After 1 day of incubation at 37°C, the cells were washed and fixed as described above and immunofluorescence labeled with dog anti-*E. chaffeensis* antibody and lissamine rhodamine-conjugated anti-dog IgG.

Total RNA isolation. Total RNA was isolated from THP-1 cells by the TRIzol method (GIBCO-BRL) (6). Briefly, infected THP-1 cells (5×10^6) were cultured as described above and harvested at 0, 3, 6, 12, 24, 48, and 72 h postinfection. The cells were pelleted by centrifugation for 5 min at 500 × *g* and lysed with 1 ml of TRIzol and repetitive pipetting, and the lysed cells were incubated for 5 min at room temperature. Chloroform (0.2 ml) was added, incubated for 3 min at room temperature, and then centrifuged at 12,000 × *g* for 15 min at 4°C. The upper aqueous-RNA-containing phase was collected, mixed with 0.5 ml of isopropanol, incubated for 10 min at room temperature, and then centrifuged at 12,000 × *g* for 10 min at 4°C. The pellet was washed once with 75% ethanol, centrifuged at 7,500 × *g* for 5 min at 4°C, and resuspended in 90 μl of diethyl pyrocarbonate-treated sterile water. The concentration of the RNA was determined by measuring the absorbance (A_{260}) with a GeneQuant II RNA and DNA calculator (Pharmacia Biotech Inc., Piscataway, N.J.). The purity of the RNA was assessed by agarose gel electrophoresis, and the remaining RNA was stored at −80°C.

cDNA synthesis (reverse transcription [RT]). Total cellular RNA (2 μg) was heated at 65°C for 3 min. After being cooled on ice, RNA was reverse transcribed in a 30-μl reaction mixture containing a reaction buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), a 0.5 mM deoxynucleoside triphosphate mixture, 1 U of an RNase inhibitor (RNasin; GIBCO-BRL)/μl, 1.5 μM oligo(dT), and 20 U of Moloney murine leukemia virus reverse transcriptase (Clontech Laboratories, Inc., Palo Alto, Calif.)/μl at 42°C for 1 h. The reaction was terminated by heating the mixture at 94°C for 2 min.

TfR mRNA semiquantitation. PCR was used to reduce nonspecific priming by the hot-start method. The cDNA (2 μl) was amplified in a 50-μl reaction mixture containing PCR buffer (10 mM Tris-HCl [pH 8.4], 50 mM KCl, 1.5 mM $MgCl_2$), a 0.2 mM deoxynucleoside triphosphate mixture, and 0.4 μM each 3′ and 5′ TfR primer (Clontech) in a DNA thermal cycler (model 480; Perkin-Elmer Corp., Norwalk, Conn.). The reaction mixture was prepared as a master mixture to minimize reaction variation, and 2.5 U of *Taq* DNA polymerase (GIBCO-BRL) was added after incubation of the mixture at 94°C for 5 min. PCR conditions involved denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 2 min. The final extension was allowed to continue for 7 min. PCR was conducted for 30 cycles to optimize TfR expression amplification within the exponential phase. To examine the relative levels of TfR mRNA expression, glucose-6-phosphate dehydrogenase (G3PDH) mRNA levels in each specimen were measured by competitive PCR (28). A G3PDH mimic DNA fragment, composed of primer template sequences identical to those of the target G3PDH cDNA to compete in primer binding and amplification, was constructed with a mimic DNA construction kit (Clontech) and primers produced at Bioserve Biotechnologies (Laurel, Md.). The G3PDH mimic DNA fragment (1 amol) was added to the PCR reaction mixture with G3PDH primers (Clontech). Since the mimic TfR DNA, constructed with the same kit, did not work reproducibly, the dose-response curve was generated with fivefold dilutions of the sample containing TfR mRNA collected at 24 h (see Fig. 3b). Based on this standard curve, the approximate amount of TfR mRNA was estimated for the time course specimens. Following RT-PCR, 9 μl of the reaction mixture was electrophoresed in 1.8% Tris-acetate–EDTA-agarose gel containing 0.5 μg of ethidium bromide/ml. ϕX174 replicative form DNA/*Hae*III fragments (0.5 μg; GIBCO-BRL), providing bands from 1,353 to 72 bp, were run in parallel. The identities of the amplified target and mimic DNA bands were determined by their predicted base pair size in the gel. The amounts of PCR products generated by the targets and mimic DNAs were analyzed by a gel video system (Gel Print 2000i; Biophotonics Corp., Ann Arbor, Mich.) and image analysis software (ImageQuant; Molecular Dynamics, Sunnyvale, Calif.). The experiment was repeated three times. The results were analyzed by Fisher's one-way analysis of variance. A photoprint of the result of a typical experiment is presented below.

RESULTS

Double immunofluorescence labeling. In order to determine in what compartment *E. chaffeensis* resides, double immunofluorescence labeling for various markers of the endosome-lysosome pathway was performed. Uninfected cells showed no labeling with anti-*E. chaffeensis* dog serum and FITC-conjugated anti-dog IgG. Infected cells showed no labeling with dog preimmune serum and FITC-conjugated anti-dog IgG (data not shown). The lack of labeling of negative controls and different patterns of labeling with various mouse monoclonal antibodies served as controls for each other. Ehrlichial inclusions were not labeled with anti-human CD63 or anti-LAMP-1 (Fig. 1), but lysosome-like structures were labeled with anti-human CD63 and anti-LAMP-1 in infected (Fig. 1) and uninfected (data not shown) cells. With secondary antibody alone (without primary antibody), no labeling was seen with LAMP-1 and CD63 (data not shown). Lysosomes contain various unique membrane glycoproteins, such as LAMP-1, LAMP-2, and CD63, which can be used as markers of lysosomal fusion with other vesicles (5). The absence of these lysosomal markers on ehrlichial inclusions indicates that ehrlichial inclusions do not fuse with lysosomes.

Ehrlichial inclusions were weakly positive and lysosomes were strongly positive for both the 57- and the 73-kDa subunits of vacuolar type H^+ ATPase and for DAMP (Fig. 1; a photo of the 57-kDa subunit is not shown). Vacuolar type H^+ ATPase is present in early and late endosomes, in lysosomes, and in portions of the Golgi apparatus and is antigenically and biochemically distinct from mitochondrial or bacterial H^+ ATPase (18). Vacuolar type H^+ ATPase is required foracidification of the endosomes and lysosomes. DAMP is a weak base containing a DNP group, which allows for easy detection with antibody to the DNP. DAMP accumulation in acidic compartments is directly proportional to the acidity of the compartment (2). Uninfected cells showed strong labeling of endosome-like structures with mouse anti-DNP and lissamine rhodamine-conjugated anti-mouse IgG and no labeling with anti-*E. chaffeensis* and FITC-conjugated anti-dog IgG. The presence of vacuolar type H^+ ATPase and the weak accumulation of DAMP within ehrlichial inclusions suggest that vacuolar type H^+ ATPase is active and that ehrlichial inclusions

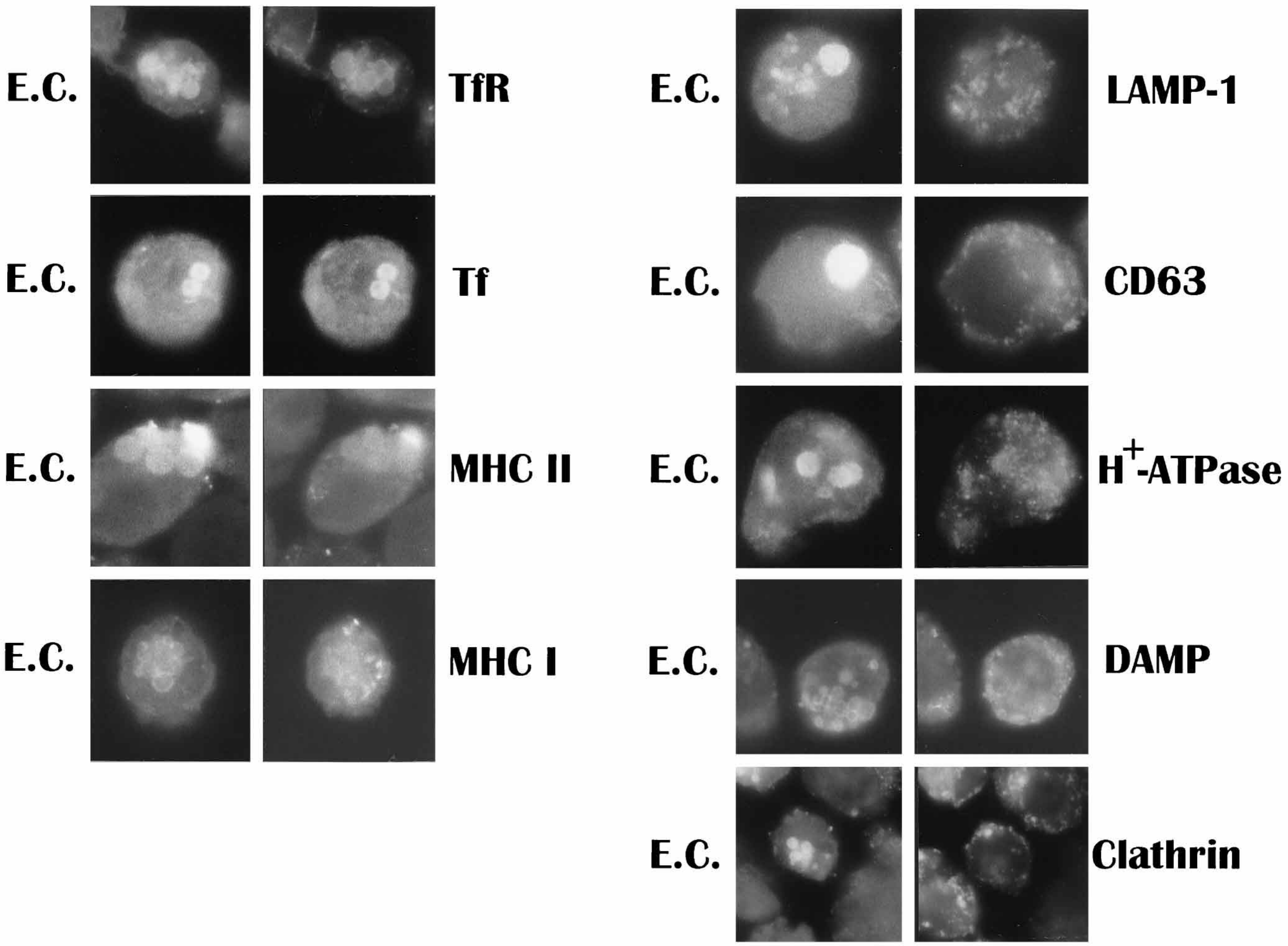

FIG. 1. Double immunofluorescence labeling of *E. chaffeensis* and various markers of endosomes. Cells of the human monocytic leukemia cell line THP-1 were infected with host-cell-free ehrlichiae and harvested at 3 days postinfection. Paired photomicrographs show *E. chaffeensis* (E.C.) on the left and membrane markers on the right. Markers are TfR, Tf:FITC-Tf (FITC-Tf was taken up by infected THP-1 cells at 2 days postinfection and was not subjected to immunolabeling), MHC class II, MHC class I, LAMP-1, CD-63, H^+ ATPase (the 73-kDa subunit of vacuolar type H^+ ATPase of bovine-brain-coated vesicles), DAMP, and clathrin. Results are representative of three independent labeling experiments. Magnification, ×788.

have a slightly acidic pH, based on their relatively weaker labeling compared to that of lysosomes.

Some ehrlichial inclusions contained the MHC class I heavy chain associated with β_2 microglobulin (approximately 30% of inclusions), β_2 microglobulin (approximately 30% of inclusions) (data not shown), and MHC class II molecules (approximately 50% of inclusions) (Fig. 1). Also, other small endosome-like structures were labeled strongly positive for MHC class I, β_2 microglobulin, and MHC class II molecules in infected (Fig. 1) and uninfected (data not shown) THP-1 cells. All inclusions were labeled strongly positive for TfR, and almost no other structures were labeled with anti-TfR (Fig. 1 and 2). However, the clathrin heavy chain was detected in small endosomes in the cytoplasm but was not detected on any ehrlichial inclusions (Fig. 1). The addition of FITC-holoTf showed that exogenous holoTf can be delivered to preformed *E. chaffeensis* inclusions (Fig. 1). These results indicate that ehrlichial inclusions are early endosomes of the Tf-TfR recycling pathway.

Time course analysis of TfR and ehrlichial colocalization. During the initial double immunofluorescence labeling experiments, it was noted that at 3 days postinfection, overall labeling with anti-TfR was much stronger in infected cells than that in uninfected cells and localized primarily in ehrlichial inclusions. This result suggests the presence of greater numbers of TfRs in infected cells than in uninfected cells. We found that at 3 h postincubation at 4°C, bound ehrlichiae and TfRs did not colocalize and few to no TfRs scattered on the cell surface or in the peripheral cytoplasm when labeling was made after saponin permeabilization. At 3 h postinfection at 37°C, some ehrlichial inclusions were negative for TfR labeling, other inclusions were weakly positive (30% of the inclusions) for TfR labeling, and few to no TfRs scattered in the peripheral cytoplasm. Between 6 and 12 h postinfection, all ehrlichial inclusions were labeled with anti-TfR and there was an increase in TfR labeling within the cytoplasm of the cell (Fig. 2), which presumably corresponded to endosomes containing TfRs. Between 12 and 24 h, all of the ehrlichial inclusions were positive for TfRs and there was a greater amount of TfR labeling within the cytoplasm. Also, between 12 and 24 h, the intensity of labeling of ehrlichial inclusions and cytoplasmic TfRs was increased (Fig. 2). Between 48 and 72 h, all TfR labeling was found in ehrlichial inclusions and with an increase in the intensity of labeling, the cytoplasmic endosomes positive for TfR

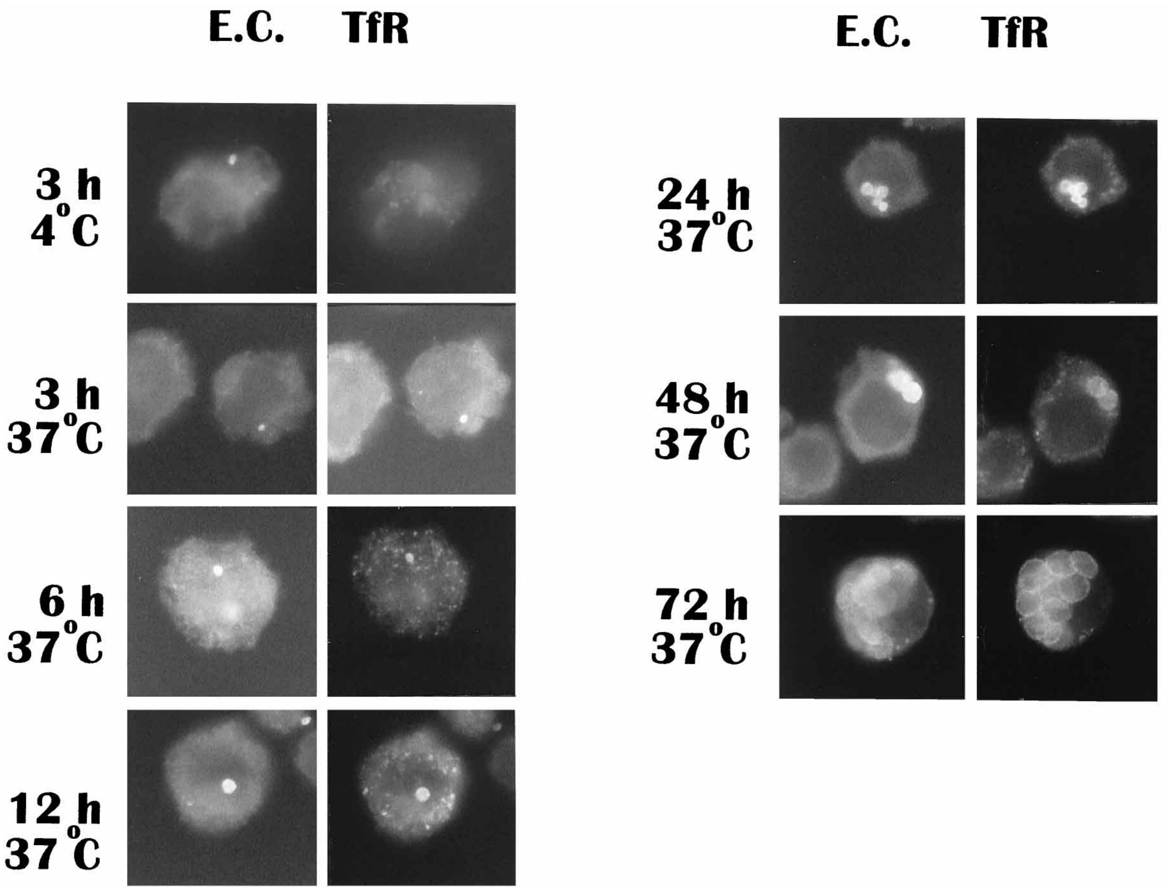

FIG. 2. Double immunofluorescence time course labeling of *E. chaffeensis* and TfRs (CD71). THP-1 cells (10^7) were infected with host-cell-free ehrlichiae and harvested after 3 h at 4°C and at 3, 6, 12, 24, 48, or 72 h postinfection at 37°C. Paired photomicrographs show *E. chaffeensis* (E.C.) on the left and TfR on the right. Results are representative of three independent labeling experiments. Magnification, ×788.

labeling disappeared (Fig. 2). Thus, ehrlichiae appear to alter their TfR numbers and traffic, including TfR recognition, delivery, sorting, and recycling to the cell surface.

TfR mRNA expression in THP-1 cells. To examine whether the increase in numbers of TfRs after *E. chaffeensis* infection is correlated with an increase in the steady-state level of TfR mRNA, RT-PCR was performed. Figure 3 shows the time course of TfR mRNA expression in THP-1 cells. Low levels of TfR mRNA were constitutively expressed in uninfected cells. The expression of G3PDH mRNA served as a control for the amounts of input RNA from the range of samples. All samples showed comparable levels of expression of G3PDH mRNA, which was confirmed by competitive PCR with the G3PDH mimic DNA. With an increase in infection time, up to 24 h postinfection, there was a gradual fivefold increase in the TfR mRNA levels compared to those at 0 h (infection) (Fig. 3). The increases in TfR mRNA at 6, 12, 24, and 48 h were statistically significant from the baseline 0-h levels. This result suggests that ehrlichiae up-regulate TfR mRNA expression.

DISCUSSION

Ehrlichia spp. survive and replicate exclusively within inclusions in monocytes and macrophages, which are primary effector cells of antimicrobial defense. Therefore, ehrlichiae must convert the hostile inclusion environment to a hospitable environment conducive not only to their survival but also to their replication. For obligatory intracellular bacteria, such as ehrlichiae, the maintenance of the inclusion environment is expected to be more stringent than with other bacteria. Various mechanisms employed by intracellular bacteria for survival are blocking of acidification of the inclusions in which they reside, blocking of lysosomal fusion with inclusions, as occurs with *M. tuberculosis* (7), escaping from the inclusion, and adapting to survival within an acidic environment. Although *E. risticii* inclusions were shown not to fuse with lysosomes (32), what type of cytoplasmic compartment the ehrlichial organisms occupied was not investigated.

We have determined that *E. chaffeensis* resides in an unique early endosome compartment. Like *M. tuberculosis* (7), *E. chaffeensis* inclusions are slow in removing MHC class I, β_2 microglobulin, and MHC class II molecules (HLA-DR and HLA-ABC). Even at 3 days postinfection, some of the ehrlichial inclusions maintained MHC class I and II molecules. MHC class I and II molecules are constitutively present on the macrophage/monocyte surface as well as in the cytoplasmic membrane compartment but are distributed differently along the endosomal-lysosomal pathway. MHC class I molecules are directed from the Golgi apparatus directly to the plasma membrane, where they are excluded during endocytosis or are rapidly recycled back to the plasma membrane after endocytosis (23). However, MHC class II molecules are directed from the Golgi apparatus to specialized MHC class II-containing endosomes and then routed to the plasma membrane (1, 25). Dur-

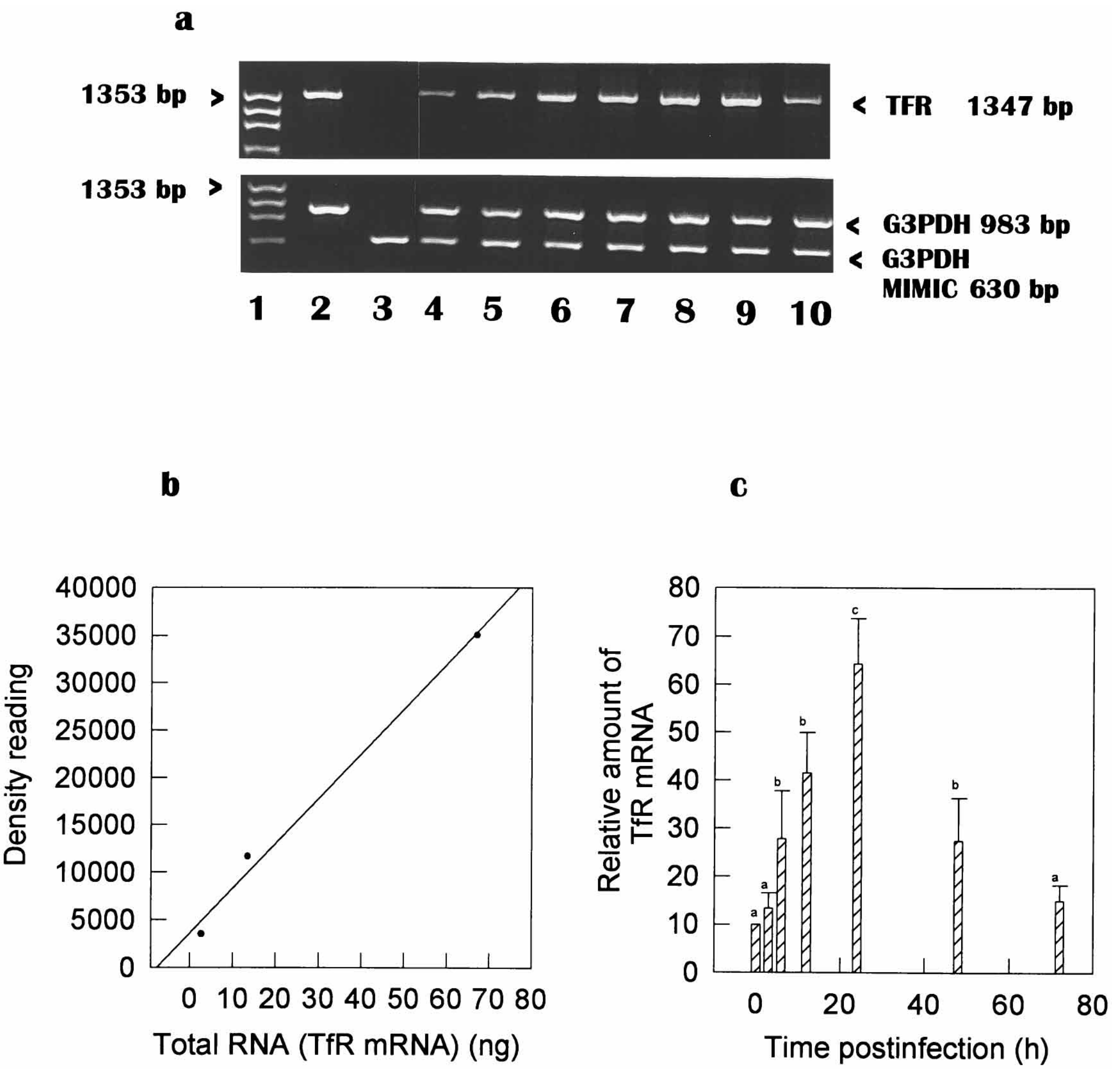

FIG. 3. TfR mRNA expression in THP-1 cells at various times postinfection. (a) THP-1 cells (10^7) were infected with sonicated *E. chaffeensis* (from 10^7 infected cells). After 0, 6, 12, 24, 48, or 72 h of infection, total RNA was extracted and cDNA was synthesized as described in Materials and Methods. A constant amount (1 amol) of the PCR mimic DNA for G3PDH was coamplified with 2 μl of cDNA, and then an aliquot of the PCR product (9 μl) was visualized on a 1.8% ethidium bromide-agarose gel. Lanes: 1, DNA ladder (ϕX174 replicative form DNA/*Hae*III fragment, 1,353 bp); 2, TfR mRNA (1,347 bp [top gel]) or the G3PDH mRNA (983 bp [bottom gel]) controls (A positive control for TfR mRNA was supplied from a human TfR control amplimer set [Clontech].); 3, the G3PDH mimic DNA control (630 bp); 4 to 10, TfR mRNA, the G3PDH mRNA, and G3PDH mimic DNA at 0, 3, 6, 12, 24, 48, and 72 h, respectively. (b) Dose-response standard curve for TfR mRNA, fivefold dilutions of cDNA collected at 24 h. The *y* axis shows sample density readings determined with image analysis software (ImageQuant). The *x* axis shows total RNA (TfR mRNA) concentrations. The correlation coefficient value (r^2) is 0.9911. (c) Approximate TfR mRNA concentrations at 0, 3, 6, 12, 24, 48, and 72 h based on the standard curve. The values are the means (bars) and standard deviations (T bars) of three independent experiments. Different letters of the alphabet indicate statistical difference ($P < 0.05$) from each other as determined by Fisher's one-way analysis of variance.

ing phagocytosis, MHC class I and II molecules are internalized in an endosome distinct from TfR endosomes (25), which do not fuse with lysosomes and are rapidly excluded from the phagosome (25). *L. pneumophila* inclusions selectively exclude MHC class I and II molecules at the stage of internalization into human monocytes (18).

We found that ehrlichial inclusions contain low levels of vacuolar type H^+ ATPase by double immunofluorescence labeling. Vacuolar type H^+ ATPase was not found in *M. avium* (29) or *Chlamydia trachomatis* inclusions (14) but was found in *Coxiella burnetii* inclusions (14). Also, there was a slight accumulation of the weak base DAMP in ehrlichial inclusions, suggesting that the vacuolar type H^+ ATPase on the inclusions is functional.

We found that *E. chaffeensis* inclusions do not colocalize with CD63 or LAMP-1, indicating that there is no fusion between the *E. chaffeensis*-containing inclusions and lysosomes, which is in agreement with our ultrastructural study of lysosomal fusion with *E. risticii* inclusions (32). This selective exclusion of fusion by ehrlichial inclusions with lysosomes is critical for the survival of an ehrlichia. LAMP-1 was found in *M. avium* inclusions (29) but not in *L. pneumophila* inclusions (7).

TfRs deliver iron to the cytoplasm through a continual cycle that shuttles the ligand Tf between the endosomal compartments and the plasma membrane. Internalization of the ligand-receptor complex is initiated via clathrin-coated vesicles, followed by delivery of Tf to the tubulovesicular network. After dissociation of iron in these acidic compartments, the apotransferrin-TfR complex is segregated from lysosomally directed molecules and directed back to the cell surface to acquire additional iron (13). Both recycling receptors, such as TfR, and receptors for lysosomally targeted ligands are internalized by the clathrin-mediated pathway and intermix in common early endosome compartments. However, these receptors diverge at sorting endosomes, while receptors for lysosomally directed molecules are targeted to late endosomes, and recycling receptors are sorted to the cell surface by a distinct class of recycling endosomes (33). It is not clear how TfRs accumulate in ehrlichial inclusions. Since the clathrin heavy chain was not detected in ehrlichial inclusions, the inclusion is also distinct from budding TfR endosomes (20). However, the ehrlichial inclusion membrane might have recognition molecules for fusion with TfR endosomes or newly synthesized TfRs directly from the Golgi apparatus might accumulate in the inclusion. The slight acidic pH of an ehrlichial inclusion may also cause prolonged retention of TfRs in the inclusion, since inhibition of acidification of endosomes was reported to delay the recycling of TfRs back to the plasma membrane (16). Ehrlichiae, therefore, may take advantage of the Tf-TfR recycling pathway to avoid lysosomal fusion.

Like *E. risticii* in murine peritoneal macrophages (24), the addition of 15 μM deferoxamine completely inhibited the survival of intracellular *E. chaffeensis* in THP-1 cells (3). Intracellular iron dependency might be a universal phenomenon among ehrlichial species. Iron is essential for ehrlichial growth, since ehrlichiae lack the glycolytic pathway, and the electron transport chain consisting of cytochrome enzymes is their sole mechanism of ATP generation (31). It is not known how *E. chaffeensis* acquires iron in the host cell. Deferoxamine chelates iron in the labile iron pool. This labile iron pool consists of iron that is immediately available to the cell for metabolic processes (15). Such iron is in a readily transportable form rather than in storage compounds, such as ferritin or hemosiderin. Iron released from endocytized Tf immediately enters this pool before it is used for metabolic processes or bound to ferritin. The fact that deferoxamine inhibits *Ehrlichia* spp. indicates that ehrlichiae, like *L. pneumophila*, derive iron from the labile iron pool (4). Our study also showed that holoTf can be delivered to preformed ehrlichial inclusions. The slight acidification of ehrlichial inclusions might be critical for the release of one iron molecule from the Tf (17) so that it might be utilized by the ehrlichiae. The selective accumulation of TfR and up-regulation of TfR at the mRNA level may be a part of a novel mechanism for iron acquisition by intracellular bacteria, such as ehrlichiae. This is the first demonstration that an intracellular bacterium can modulate the host cell expression of TfR, a critical protein required by the host cell. Further studies of the mechanism of up-regulation of TfR mRNA and the mechanism of iron acquisition by ehrlichiae are under way.

ACKNOWLEDGMENTS

This research was supported by grant AI30010 from the National Institutes of Health.

The anti-H4A3 monoclonal antibody developed by James E. K. Hilfreth and J. Thomas August was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Md., and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, under contract N01-HD-2-3144 from the NICHHD.

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Editor: P. E. Orndorff