

Detection of ehrlichial infection by using polymerase chain reaction

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Abstract

The ehrlichiae are small Gram-negative obligatory intracellular bacteria in the family Anaplasmataceae. Ehrlichiae infection in an accidental host may result in severe fatal diseases such as human monocytotropic ehrlichiosis (HME), an emerging, tick-borne disease. Using *Ehrlichia muris* as a model organism, here I show that *E. muris* can infect bone marrow-derived dendritic cells and macrophages. The ehrlichial infection can be detected by polymerase chain reaction in cultured dendritic cells and macrophages as well as experimentally infected animals. (J Med Life Sci 2010;7:110-113)

Key Words : Ehrlichia muris, Bacterial infections, Dendritic cells, Macrophages, Polymerase chain reaction

Introduction

Human monocytotropic ehrlichiosis (HME) is an emerging, tick-borne disease caused by infection of the obligatory intracellular bacterium, *Ehrlichia chaffeensis*, which is a NIAID Category C priority pathogen^{1, 2)}. *E. chaffeensis* infection in human with delayed or ineffective treatment may be fatal, particularly in immunocompromised hosts^{3, 4)}. Ehrlichiae frequently cause a persistent infection in their natural hosts which seem to have proper immune defense mechanism to specific ehrlichia strains. Such examples include *E. canis* in dogs, *E. chaffeensis* in deer, *E. muris* in mice and *E. ruminantium* in sheep, goats or cattle^{1, 5)}.

However, ehrlichiae infection in accidental hosts may result in severe fatal disease, similar to toxic shock-like disease, as seen in *E. chaffeensis* infection in human or infection of IOE Ehrlichia strain in mice⁶⁻⁸⁾. The ehrlichiae are small Gram-negative obligatory intracellular bacteria in the family Anaplasmataceae and reside in endosomal compartments, primarily in macrophages or monocytes. The ehrlichiae lack genes for the biosynthesis of LPS and peptidoglycan, which can elicit innate immune response^{9, 10)}. *E. muris*, a natural pathogen in mice, is closely related to *E. chaffeensis* and is frequently used as an Ehrlichia infection model in mice¹¹⁾. Infection of *E. muris* in laboratory strain of mice such as BALB/c, AKR and C57BL/6 results in splenomegaly, anemia and spleen follicular hyperplasia with

subsequent development of a low-level persistent infection^{11, 12)}. Both CD4 and CD8 T cells play important roles in Ehrlichia immune response^{13, 14)}.

In the present study *E. muris* was found to infect bone marrow-derived dendritic cells and macrophages. The ehrlichial infection can be detected by polymerase chain reaction in cultured dendritic cells and macrophages as well as experimentally infected mice.

Material and Methods

Mice

C57BL/6 mice were from Taconic Farm, Inc. Mice were maintained under specific pathogen-free conditions. All mice were maintained and used in accordance with institutional and National Institutes of Health guidelines.

Bacterial stocks and in vitro Ehrlichia infection of cells

E. muris, monocytotropic ehrlichial strain (ATCC VR-1411) was used in this study. *E. muris* was cultivated in DH82 cells with 5% FBS-supplemented DMEM at 37°C. Ehrlichiae were harvested when 90-100% of the cells were infected, and stored -80°C in sucrose-phosphate-glutamate buffer (0.218 M sucrose, 3.8 mM KH₂PO₄, 7.2 mM K₂HPO₄, and 4.9 mM monosodium glutamic acid, pH 7.0), and used for in vitro infection. The cells were cultured without antibiotics and *E. muris* bacterial stock was added to the cells. Infected cells were stained by Diff-Quick (Imeb, Inc) or Hoechst 33342 (Invitrogen) staining. In order to produce virulent infectious stocks for in vivo infections, live mice were used

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as described previously¹². Briefly, C57BL/6 mice were inoculated i.p. with 0.5 ml of a 10⁻¹ dilution of the frozen stock. On-day 9 after inoculation, the mice were sacrificed, the spleens were harvested, and the homogenate was suspended in sucrose phosphate-glutamate buffer. Large particles of debris were removed by centrifugation at 500 g for 5 min, and the supernatant was then aliquoted and stored at -80°C as a 10⁻¹ stock of *E. muris*.

Bone marrow-derived macrophages

Bone marrow-derived macrophages were prepared as described¹⁵. Briefly, bone marrow from tibia and femur was obtained by flushing with DMEM (Invitrogen). The medium was DMEM supplemented with 10% heat-inactivated FBS, glutamine (both from Invitrogen) and 30% L929 cell supernatant containing M-CSF. Bone marrow cells were cultured in 10 ml at an initial density of 1 × 10⁶ cells/ml in 100 mm petri dish (Becton Dickinson) at 37°C in humidified 10% CO₂ for 6 days. Cells were harvested with cold PBS (Invitrogen), washed, resuspended in DMEM supplemented with 10% FBS and used at a density of 2 × 10⁵ cells/ml for experiments unless mentioned otherwise.

Bone marrow-derived dendritic cells

Dendritic cells (DCs) were grown from wild-type as described previously¹⁶. Briefly, bone marrow from tibia and femur was obtained as described above, and bone marrow cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 50 μM of 2-ME, and 2 mM of glutamine supplemented with 3% J558L hybridoma cell culture supernatant containing GM-CSF. The culture medium containing GM-CSF was replaced every other day. At day 6 of culture, nonadherent cells and loosely adherent DC aggregates were harvested, washed, resuspended in RPMI 1640 supplemented with 5% FBS and used at a density of 2 × 10⁵ cells/ml for experiments unless mentioned otherwise.

Ehrlichia challenge of mice in vivo

In vivo *E. muris* infection method was established previously using *E. muris* stock prepared from the spleen of the infected mice¹². Briefly, age (6–8 weeks old) and sex matched groups of mice were infected i.p. with *E. muris* with a 10⁻¹ dilution of the *E. muris* stock. On the indicated days of infection, mice were sacrificed, and immune responses were assessed. Selected organs were harvested for histology, and bacterial loads were determined by PCR.

Samples of spleen were fixed with 10% formalin and stained by hematoxylin and eosin (H&E) for histopathological examination.

Determination of Ehrlichia bacterial load in tissues and cells

Since *E. muris* is an obligatory intracellular bacterium that does not grow outside of the cells, quantification method for the Ehrlichia load was established using PCR. Briefly, the ehrlichial load was determined by PCR of the Ehrlichia dsb gene (GenBank accession number: AY236484), which encodes a thio-disulfide oxidoreductase or disulphide bond formation protein of *E. muris*. Primer sequences are as follow: *E. muris* forward, TTCTTTGACTATTCCTGTGGCTAC; *E. muris* reverse, AATGACGCCTCACCGAGTAT; mouse GAPDH forward, CAACTACATGGTCTACATGTTTC; and GAPDH reverse, CTCGCTCCTGGAAGATG. The substrate for amplification was DNA purified from samples using the DNeasy Tissue kit (Qiagen, Valencia, CA). PCR was performed using the 7300 real time PCR system from Applied Biosystems. Results were normalized to GAPDH levels in the same sample.

Results and Discussion

In order to find the major cell type for *E. muris* infection, we generated bone marrow-derived DCs and macrophages from C57BL/6 mice and challenged them with *E. muris* in vitro. *E. muris* infection caused generation of bacteria-containing vacuoles (morulae) in both DCs and macrophages as observed by Diff-Quick staining (Fig. 1). Diff-Quick staining showed that there was massive multiplication of *E. muris* and almost all of the cells were infected with *E. muris*. Furthermore, *E. muris* proliferated

Figure 1. *E. muris*-infected cells (5 days postinfection) were stained by Diff-Quick staining. Magnification: 1,000× DC: dendritic cell, MΦ, macrophage.



efficiently in both DCs and macrophages, as quantified by quantitative PCR using primers specific for the *E. muris* dsb gene (Fig. 2). Collectively, these data suggest that DCs as well as macrophages can serve as major reservoirs of *E. muris* infection.

To find whether Ehrlichial infection can be detected in experimentally infected mice, C57BL/6 mice were infected with *E. muris* by i.p. inoculation and bacterial loads in the blood and splenic tissues were assessed by quantitative PCR using primers specific for the *E. muris* dsb gene (Fig. 3). C57BL/6 mice showed increased amount of *E. muris* bacterial loads in the blood on day 7 and 9 post-infection (Fig. 3B). In the spleen, *E. muris* load peaked on day 9 and decreased gradually from day 10 to day 14 post-infection in the mice.

These data show that dsb DNA of *E. muris* can be detected in cultured primary cells as well as in infected animals at the acute stage.

Figure 2. Bone marrow-derived dendritic cells (BMDC) and macrophages (BMDM) from wild-type mice were infected with *E. muris* in vitro for the indicated time period. Bacterial loads in infected cells were quantified by PCR using primers specific for *E. muris* dsb gene.

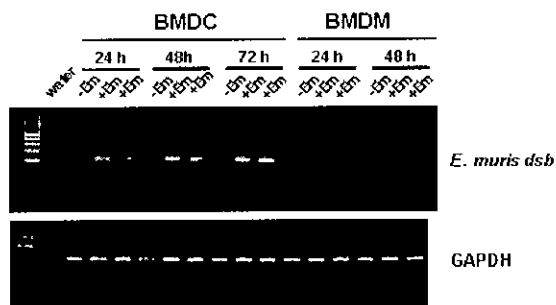
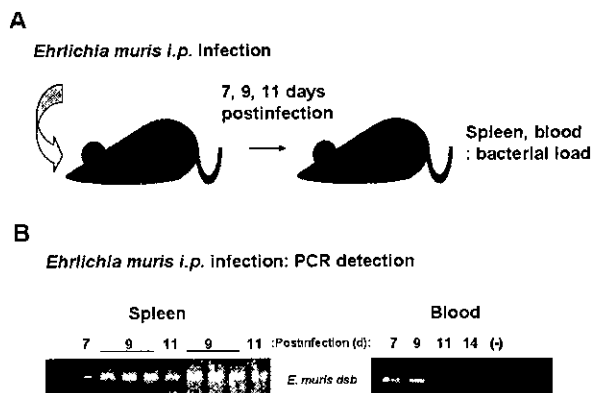


Figure 3. (A) C57BL/6 mice were infected with *E. muris* i.p. for the indicated time period. (B) *E. muris* loads in blood and spleen were quantified by PCR using primers specific for *E. muris* dsb gene.



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