

SURVIVAL AND REPLICATION OF *ESCHERICHIA COLI* O157:H7 INSIDE THE MICE PERITONEAL MACROPHAGES

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Submitted: February 12, 2007; Returned to authors for corrections: October 17, 2007; Approved: January 20, 2008.

ABSTRACT

The replication of *Escherichia coli* O157:H7 on the resident peritoneal macrophages of four mice strains (BALB/c, CD1, C57BL, and Swiss) has been investigated. Macrophagial bactericidal killing activity was estimated via studying their ability to internalize (gentamicin-protected) *E. coli* during 2, 4, 24, and 48 h assays. Host genetic background has been found to show no significant effect on the ability of resident peritoneal macrophages to kill *E. coli* O157:H7.

Key words: *E. coli* O157:H7, macrophages, mice, gentamicin.

Escherichia coli O157:H7 is commonly considered the most infectious bacteria causing bloody diarrhea and acute kidney failure in children that could develop into a hemolytic uremic syndrome. Enterohaemorrhagic *E. coli* (EHEC) comprise a group of emerging zoonotic pathogens of worldwide importance (2).

EHEC produces several recently described virulence determinants, which enables it to colonize the large bowel and cause the disease (25). Many of its virulence factors are secreted by a type III secretion system which delivers virulence factors directly into host cells (12). Another potential virulence factor is O157:H7 LPS which enhances the cytotoxicity of verotoxin or Shiga-like toxin (Stx) on human vascular endothelial cells *in vitro* (15). However, its effects *in vivo* require more investigations, and therefore researches are undoubtedly necessary in order to determine the best cell model, that could be used *in vivo* to explore the effects of these bacteria (21). However, the experimental data indicate that the reactive oxygen intermediates (ROIs) production represents one of the primary mechanisms conducted by host macrophages for limiting the replication of bacteria (6). It is well documented that the opsonization of the bacteria by immunoglobulin G (IgG) and the activation of macrophages by gamma interferon (IFN- γ), are known factors that bring about an increase in the ROI production and bactericidal activity (9, 17). The purpose of this

article is to: (i) demonstrate the net intracellular replication; (ii) assess the ability of *E. coli* O157:H7 strain to resist inside mice macrophages; and (iii) determine the concentration of the gentamicin that eliminates the extracellular bacteria, without affecting intracellular bacteria.

Mice strains

Specific-pathogen-free 8-weeks-old female BALB/c, C57BL/6 (Inbred), CD-1, and Swiss (Outbred); purchased from Charles River Laboratories (France); were used. Groups of 7 or 8 mice were housed in polypropylene cages with sterilized bedding under controlled conditions; temperature ($24 \pm 1^\circ\text{C}$), and relative humidity (55%); and maintained on a standard diet and sterilized water. Water bottles were replaced daily.

Bacterial strains

E. coli (serotype O157:H7) colonies, clinically isolated from stool samples of a patient with hemorrhagic colitis, were collected during an outbreak of the disease in Damascus, Syria, in 2001. Ten well-isolated colonies that are typical for *E. coli* O157:H7 (i.e.: colorless, nonsorbitol fermenters) were selected and bio-chemically identified to be *E. coli*. The isolates were then confirmed by agglutination with *E. coli* O157:H7 antiserum coated latex test (Oxoid, UK) to ascertain O157 antigen presence. Positive colonies were sub-cultured on tryptic soy agar (Merck,

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Germany) and incubated for overnight at 37°C, then screened by H7 latex test (Oxoid, UK).

Bacterial growth conditions

The bacteria were grown with agitation (100 rpm) in Luria broth (LB), (Q-Biogen, Cedex, France) at 37°C for 6 h using 2% of an overnight culture, bacteria were harvested by centrifugation (6,000 × g for 10 min), and re-suspended in sterile phosphate-buffered saline (PBS, pH 7.4). The bacterial concentration (CFU) was estimated by optical density (OD) value at 600 nm and adjusted to the desired concentration of 10⁹ CFU/ml.

Survival and replication of the *E. coli* strains in cultured murine macrophages

Six to 8-weeks old female BALB/c, C57BL6J, CD1, and Swiss mice were euthanized by isoflurane overdose. Immediately following euthanasia, cells from the peritoneal cavity were harvested by washing using 8 ml of Dulbecco's minimal essential medium (DMEM), (Biochrom AG, Berlin, Germany) supplemented with 5% fetal calf serum (FCS), (Eurolone, UK) and 5 U/ml heparin. Total cell numbers and viability were determined using the [0.0003% wt/vol] acridine orange, (Caledon, Canada) with ethidium bromide (Eurolone, UK) [0.001% wt/vol] exclusion technique. Explanted cells were then cultured at a density of 10⁵ cells per well in sterile 24-well micro plates (TPP, Switzerland) in DMEM supplemented with 5% FCS and 5 µg/ml gentamicin (Biochrom AG, Berlin, Germany). After overnight incubation at 37°C in 5% CO₂, cell cultures were enriched with macrophages by eliminating non-adherent cells. Cells were infected either with *E. coli* O157:H7, or with *E. coli* DH5a at a multiplicity of infection of 100 bacteria per cell for 45 min at 37°C to allow the phagocytosis of the *E. coli*. After 45 min, free cell culture media (without FCS) were removed and replaced by DMEM supplemented with 5% FCS and 50 µg/ml of gentamicin, then incubated at 37°C with 5% CO₂ continued for 1 h to kill all remaining extracellular *E. coli*. The macrophages were then washed with PBS supplemented with 5% FCS and maintained thereafter in DMEM with 5% FCS and 5 µg/ml of gentamicin. Cell culture media were replaced with fresh media every 24 h. Immediately after plating, parallel experiments were performed to examine the effects of cultured macrophages treated with either (i) 25 U/ml of IFN-γ (PAN, Biotech, Germany); (ii) the mice anti-*E. coli* serum; or (iii) a combination of IFN-γ and mice anti-*E. coli* serum, on the capacity of phagocytes to kill intracellular *E. coli* O157:H7.

Intracellular viability assay of *E. coli* strains in macrophages

Macrophages and *E. coli* were grown in a prepared medium for 30 min, the lamina wells were washed several times with Hanks solution, and 200 µl of the growing medium were added. This medium contains 10% FCS with 5, 10, 50 or 100 µg/ml of gentamicin. Two, 24, and 48 h after infection, macrophages were washed with PBS supplemented with 5% FCS and lysed with

0.2% Triton X-100. Five minutes after incubation at room temperature, serial 10-fold dilutions of the lysates were prepared in sterile PBS and plated on LBA and incubating at 37°C for overnight. Plating was performed in triplicate from 3 wells per strain each time. Data obtained were expressed as log₁₀ intracellular *E. coli*.

Statistical analysis

Data were analyzed using the Student's two-tailed *t* test (Abacus Concepts, 1994). All statistical comparisons were considered significant at the 5% probability level.

The presence of gentamicin at 5 or 10 µg/ml concentrations has no effect on the counts of the intracellular bacteria after 2 or 4 h of incubating periods, compared with cultures incubated without antibiotic. In contrast, the presence of gentamicin at 50 or 100 µg/ml concentrations decreased the intracellular bacterial counts, for both incubation times, by 20 to 30 folds. Therefore, a dose of 5 µg/ml of gentamicin was used to incubate the macrophages and *E. coli*, because it eliminates the extracellular bacteria without affecting intracellular bacteria.

Fig. 1 shows that the *E. coli* O157:H7 bacterial colonies were slightly increased in C57BL strain compared to BALB/c strain particularly after 2 h of post-infection ($p < 0.0005$). The bacterial count in these two strains was identical for the *E. coli* O157:H7 ampicillin-resistant or susceptible strains.

The bacterial count, 2, and 24 h after infection, was slightly higher in Swiss strain compared with CD1 strain (Fig. 2). In contrast, after 4 h the bacterial count was higher in CD1 than the Swiss mice ($p < 0.05$) (Fig. 2). However, it remained unchanging in the other mice strains (BALB/c, Swiss, CD1 and C57BL), after 48 h of infection (Figs. 1, 2). On the other hand, the bacterial count increased in accordance with the time of macrophage infection in all mice strains, but the lethal effect of macrophage reversely decreased to bacterial exposure time.

The data concerning the lethal effect of macrophages did not reveal any differences in bacterial counts either before or after adding the serum containing antibodies against the *E. coli*, or the gamma interferon (data not shown).

Ampicillin-resistant *E. coli* O157:H7, and *E. coli* DH5a which is a non pathogenic strain, did not both stimulate the macrophages apoptosis 24 h after infection in all mice strains (data not shown). Macrophage is considered the major constituent of the natural immunity system. It can specify the antigens in large groups of bacteria and differentiate it from self antigens, and can also activate the procedures of bacterial elimination within host body. The phagocytic response of innate immune cells such as macrophages is defined by the activation of complex signaling networks that are stimulated by microbial contact (13). Phagocytosis has served as the classic model of microbe-innate immune interactions, and enormous progress has been made toward understanding the consequences of this interaction (10).

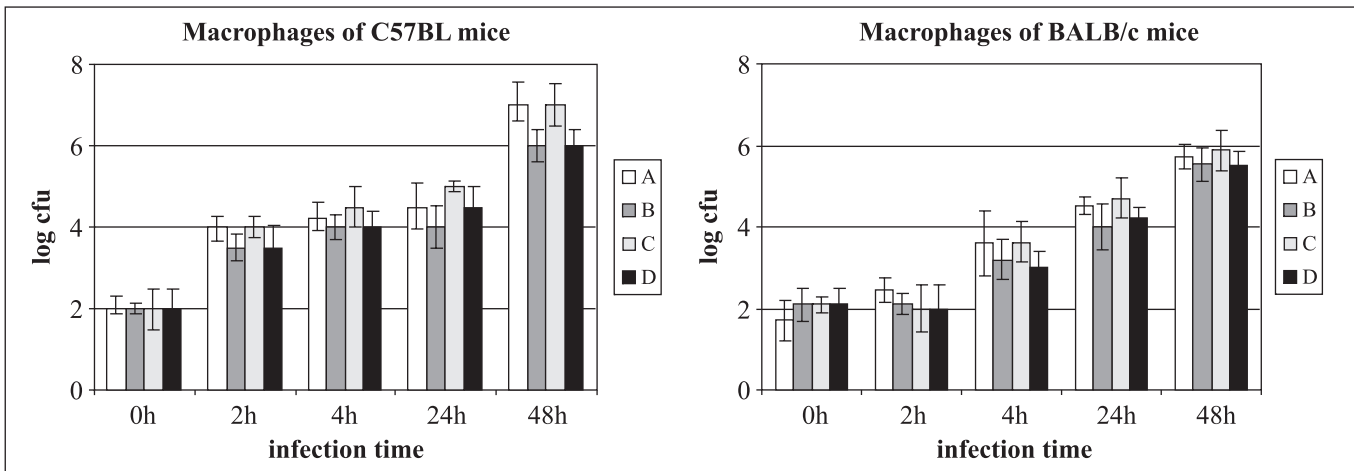


Figure 1. Intracellular survival and replication of *E. coli* O157:H7 in primary cultures of resident peritoneal macrophages from inbred mice. A and B: *E. coli* O157:H7 susceptible to ampicillin (A. untreated macrophages, B. macrophages treated with 5 µg/ml gentamicin). C and D: *E. coli* O157:H7 resistant to ampicillin (C. untreated macrophages, D. macrophages treated with 5 µg/ml gentamicin). The error bars represent the standard deviation in an average of log₁₀ intracellular *E. coli* O157:H7 from triplicate plating taken at each time.

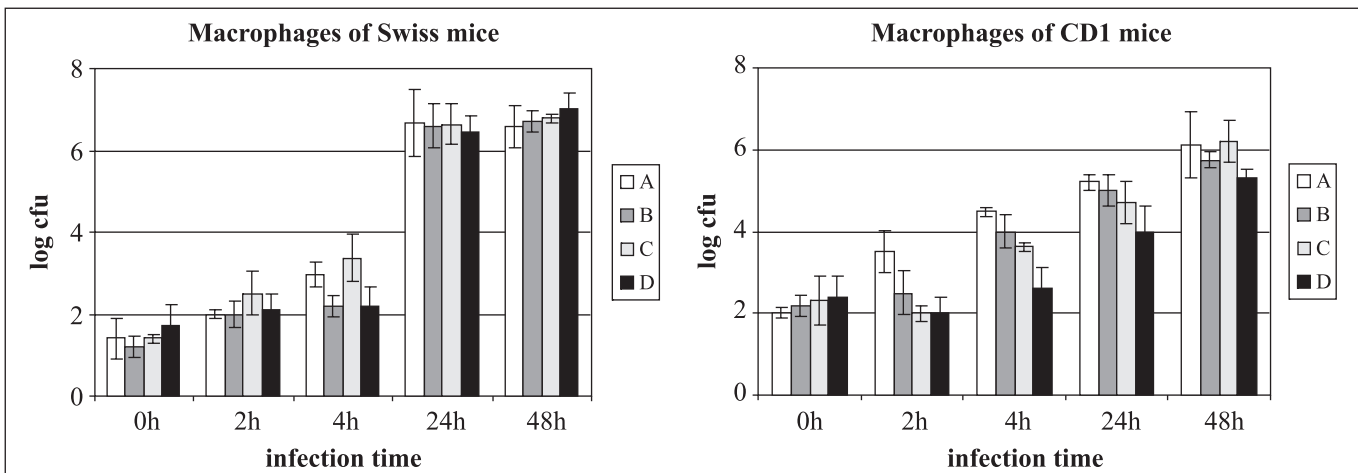


Figure 2. Intracellular survival and replication of *E. coli* O157:H7 in primary cultures of resident peritoneal macrophages from outbred mice. A and B: *E. coli* O157:H7 susceptible to ampicillin (A. untreated macrophages, B. macrophages treated with 5 µg/ml of gentamicin). C and D: *E. coli* O157:H7 resistance to ampicillin (C. untreated macrophages, D. macrophages treated with 5 µg/ml of gentamicin). The error bars represent the standard deviation in an average of log₁₀ intracellular *E. coli* O157:H7 from triplicate plating taken at each time.

Toll receptors enable the macrophage to recognize the microbial antigen, and by means of the TLR4 it can recognize the LPS (7). Moreover, TRL1-9 receptors that are identical in mice and humans can also recognize viral, bacterial, fungal and parasitic organisms (24).

The way in which the bacteria interacts with eukaryote cells *in vitro* (inside or outside the cells), was well studied. Antibiotics

and bacteriophages which destroy the bacteria were used to eliminate the extracellular bacteria (20). Elsinghorst (4) reported that gentamicin, could kill the extracellular bacteria without affecting the intracellular ones; but recently Oyha *et al.* (19) demonstrated that this antibiotic might cross into the cellular membrane and affect the intracellular bacteria. Nevertheless, in our present work, 5 µg/ml of gentamicin was the concentration

which eliminates the extracellular bacteria without affecting the intracellular ones.

In this study it turned out that, when the minimum effective dose of gentamicin is used, the intracellular bacteria do not change (Figs. 1 and 2). The bacteria isolated from peritoneal macrophage 72 h after infection, was about 10^9 CFU in all mice strains, so the gentamicin dose was not enough to eliminate the extracellular bacteria, 48 h after infection.

To understand the role of genetic and cellular agents in the innate immune system, the bacterial and the host factors which affect the nonopsonic phagocytosis were studied. Nonopsonic phagocytosis is the mechanism which participates in the killing process of the bacteria by the macrophage in the absence of any immunity serum (18). Whereas, the phagocytosis with opsonization mechanism is a different killing process characterized by the high percentage of bacterial death in presence of antibodies against the microbe (1). Differences in bacterial counts after infecting the macrophage without opsonization have not been observed suggesting that the macrophage genes differences do not affect the killing ability of *E. coli* O157:H7. Similar results were reported by Hamric *et al.* (5), who used macrophages from BALB/c, CD1, C57BL, C3H/HeG and C3H/HeN, and two strains of *E. coli* K12 one of which has FimH⁺ and the other has only the recessive gene FimH⁻. Moreover, Sukumaran *et al.* (22) observed that opsonization, with either complement proteins or antibody is not required for uptake and survival of the bacteria within the macrophages.

Pathogens have developed numerous strategies to escape the deleterious effect of phagocytosis by professional phagocytes among which are evading the phagocytosis, killing the phagocytes or surviving inside them (8). These are the most common processes reported so far, and some of such mechanisms include preventing recognition by phagocytic receptors or blocking the uptake by professional phagocytes. Experiments conducted by Celli *et al.*, have demonstrated that the EPEC uptake by macrophages did not involve opsonin-dependent phagocytic receptors due to the inhibition of phosphatidylinositol (PI) 3-kinase-dependent F-actin rearrangements that are required for bacterial uptake (3). Furthermore, the latter process could exert inhibitory effects on macropinocytosis as reported by Murray *et al.*, (16). In this context, our findings have accordingly shown that *E. coli* O157:H7 bacteria utilised a similar mechanism to survive and replicate inside the mice macrophages.

Results showed that *E. coli* O157:H7 not only extends the survival of murine infected macrophage, for all mice strains used in this study, but also renders them resistant to apoptosis. Moreover, the macrophages infected with *E. coli* O157:H7, have been revealed to be resistant to DNA fragmentation exposure. Our findings corroborate previous results that *E. coli*, induced cell proliferation of mammary cell cow and that OmpA *E. coli* K1 inhibit the apoptosis of human and murine infected macrophage

Long *et al.* (14), Sukumaran *et al.* (23). However, another study found that Stx1 and Stx2 produced by *E. coli* induced apoptosis of human mucosal epithelial cells that express the Gb3 receptor for the toxins (11).

Further studies ought to be achieved in order to understand the role of the resident peritoneal macrophage in bacterial killing mechanisms, and the interaction mechanisms between *E. coli* O157:H7 and the macrophages.

ACKNOWLEDGMENTS

The author thanks the Director General of AECS and the Head of the Molecular Biology and Biotechnology Department for their support. He thanks also Dr. M. Safi for his critical reading of this manuscript.

RESUMO

Sobrevivência e multiplicação de *Escherichia coli* O157:H7 no interior de macrófagos peritoniais de camundongos

A multiplicação de *Escherichia coli* O157:H7 em macrófagos peritoniais residentes de quatro linhagens de camundongos (BALB/C, CD1, C57BL e Swiss) foi investigada. A capacidade bactericida dos macrófagos foi estimada através da avaliação de sua capacidade de internalizar *E. coli* (protegidos pela gentamicina) em ensaios de 2, 4, 24 e 48h. Observou-se que as características genéticas do hospedeiro não têm efeito significativo na capacidade dos macrófagos peritoniais eliminarem *E. coli* O157:H7.

Palavras-chave: *Escherichia coli* O157:H7, macrófagos, camundongos, gentamicina

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