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AIMS AND SCOPE

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EDITORIAL CORRESPONDENCE should be addressed to

Acta virologica Editorial Office
Institute of Virology
Slovak Academy of Sciences
Dúbravská cesta 9
842 46 Bratislava
Slovak Republic
Telephone (internat.): +421-7-59413124
Fax: +421-7-54776380, +421-7-54774284
E-mail: viruacta@nic.savba.sk

Publisher: SAP – Slovak Academic Press Ltd
P.O. Box 57
810 05 Bratislava 15
Slovak Republic
Telephone: +421-7-55565862
Fax: +421-7-55421729

INDUCED PRODUCTION OF NITRIC OXIDE AND SENSITIVITY OF ALVEOLAR MACROPHAGES DERIVED FROM MICE WITH DIFFERENT SENSITIVITY TO *COXIELLA BURNETII*

K. YOSHIIE*, S. MATAYOSHI, T. FUJIMURA, N. MAENO, H. ODA

Department of Bacteriology, Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8520, Japan

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Summary. – We compared *in vitro* sensitivities to *Coxiella burnetii* of alveolar macrophages, derived from mice sensitive and resistant to *C. burnetii*, respectively, and examined the role of nitric oxide (NO) in the *C. burnetii* infection. Alveolar macrophages of sensitive A/J mice showed a larger population of *C burnetii* antigen-positive cells than those of resistant C57BL/6 mice. *C. burnetii* induced NO production in alveolar macrophages, but N-methyl-L-arginine and sodium nitroprusside (SNP), NO inhibitor and donor, respectively, did not inhibit the infection. Thus the NO induction seems to be independent of the cell defense mechanism against the *C. burnetii* infection.

Key words: Coxiella burnetii; alveolar macrophage; nitric oxide; in vitro sensitivity

Introduction

Coxiella burnetii is an obligatory intracellular parasite, which infects humans and animals. There are many strains of *C. burnetii* with different virulence (Kazár, 1988; Kazár *et al.*, 1993; Vodkin *et al.*, 1986) causing an acute form of Q fever or chronic forms of endocarditis, granulomatous hepatitis etc. in humans (Hendrix *et al.*, 1991; Samuel *et al.*, 1985; Tissot-Dupont *et al.*, 1992). An endemic of Q fever in humans occurred by inhalation of contaminated aerosols (Marrie, 1996). When the pathogen is inhaled in a form of aerosol, alveolar macrophages might have an important role in defense against *C. burnetii* (Hall *et al.*, 1981; La-Scola *et al.*, 1997). Murine pneumocytes can destroy *C. burnetii*, and at the same time, *C. burnetii* destroys some pneumocytes in a murine model of nasal infection (Khavkin and Tabibzadeh, 1988). *C. burnetii* can survive this defensive step by spreading throughout the host. Experimental infection by the intraperitoneal route with Nine Mile strain revealed that there are several strains of mice with different sensitivity to *C. burnetii* infection; sensitive A/J, moderately sensitive BALB/c and resistant C57BL/6 strains (Scott *et al.*, 1978; Williams *et al.*, 1985). In this route of infection, intraperitoneal macrophages might be the first place for the pathogen to proliferate. These peritoneal cells, like alveolar macrophages, may have a role of limiting the *in vivo* propagation of *C. burnetii*. As peritoneal and alveolar macrophages are located in different organs and have different functions, it is unclear whether the sensitivity of mice observed *in vivo* is based on the sensitivity of individual macrophages in each organ.

It is also unclear what mechanism(s) destroys *C. burnetii* in macrophages. NO inhibits the growth of *Rickettsia conorii* and *R. prowazekii* in fibroblasts and macrophages (Feng *et al.*, 1994; Turco and Winkler, 1993; Walker *et al.*, 1997). Induced NO production takes place in alveolar macrophages infected with several kinds of microbes (Denicola *et al.*, 1993; Goodrum *et al.*, 1994; Jeevan *et al.*, 1995; Yamamoto *et al.*, 1996; Zhang and Morrison, 1993). The antimicrobial

*E-mail: MXB01623@nifty.ne.jp; fax: +8199-2645643.

Abbreviations: i.p. = intraperitoneal(ly); MOI = multiplicity of infection; NMMA = N-methyl-L-arginine; NO = nitric oxide; PBS = phosphate-buffered saline; p.i. = post infection; SNP = sodium nitroprusside

effect of NO is dependent on the kind of pathogen. In this study, we infected alveolar macrophages derived from sensitive A/J and resistant C57BL/6 mice with the Nine Mile strain of *C. burnetii,* and investigated their sensitivity *in vivo* and *in vitro*. We also compared the induced NO production by alveolar macrophages and the effects of an NO inhibitor and donor on the infection

Materials and Methods

Rickettsia. The Nine Mile strain of *C. burnetii* was provided gently by Dr. K. Hirai, Department of Veterinary Microbiology, Faculty of Agriculture, Gifu University, Gifu, Japan. This strain was propagated in embryonated eggs and L929 cells. *C. burnetii*-infected L929 cell suspensions and yolk sac homogenates were stored at -80°C. *C. burnetii* in aliquots of an infected L929 cell suspension was inactivated by irradiation with ^{60}Co. The inactivated pathogen could not grow in embryonated eggs and L929 cells.

Mice. Sensitive A/J, intermediately sensitive BALB/c and resistant C57BL/6 strains of mice were purchased from Kuroda Animal Breeding Inc. (Kumamoto, Japan). Female mice (6–10-week-old) were used for alveolar lavage.

Alveolar macrophages After euthanasia with sodium nembutal (Shionogi Co. Ltd., Tokyo, Japan), an elastic tube was inserted into mouse trachea, and bronchoalveolar space was lavaged with 10 ml of warm Dulbecco's Minimum Essential Medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% of fetal calf serum, 1000 IU/ml penicillin and 1000 μg/ml streptomycin (Bio-Whittaker, Walkersville, MD, USA). The collected cells were centrifuged and resuspended in the same medium. After overnight preincubation in a 15 ml polystyrene centrifuge tube for cell adhesion, the noncontaminated tubes were selected and used in further experiments. The antibiotics-free medium was used after this step throughout the experiments. Lavaged alveolar macrophages were infected at the multiplicity of infection (MOI) of 10 to 1000 rickettsial particles per cell. After adsorption of the pathogen for 1 hr, alveolar macrophages were washed twice and incubated in 5% CO_2 at 37°C. Then the infected cells were harvested at various intervals. The cells were detached by using an EDTA-trypsin solution and were washed twice with phosphate-buffered saline (PBS) by centrifugation. The pooled pellets were resuspended in 0.2 ml of PBS, and 5 ml of chilled methanol (-30°C) was added for fixation.

Antibodies. An antiserum against *C. burnetii*, used as primary antibody, was obtained from mice infected with *C. burnetii* at day 35 post infection (p.i.). FITC-conjugated anti-mouse-IgG goat IgG (Caltag Laboratories Inc., Burlingame, CA., USA) was used as secondary antibody.

Flowcytometry. The fixed cells were washed twice with PBS by centrifugation, resuspended in 50 μl of PBS, and the same volume of the primary antibody was added and reacted for 30 mins at 37°C. The cells were washed twice with PBS again, and the secondary antibody was added. After 30 mins of reaction, the cells were washed twice with PBS. Fluorescence intensity and population of *C. burnetii* antigen-positive cells were measured by an EPICS Elite flowcytometer (Beckman-Coulter Inc., Fullerton, CA, USA.).

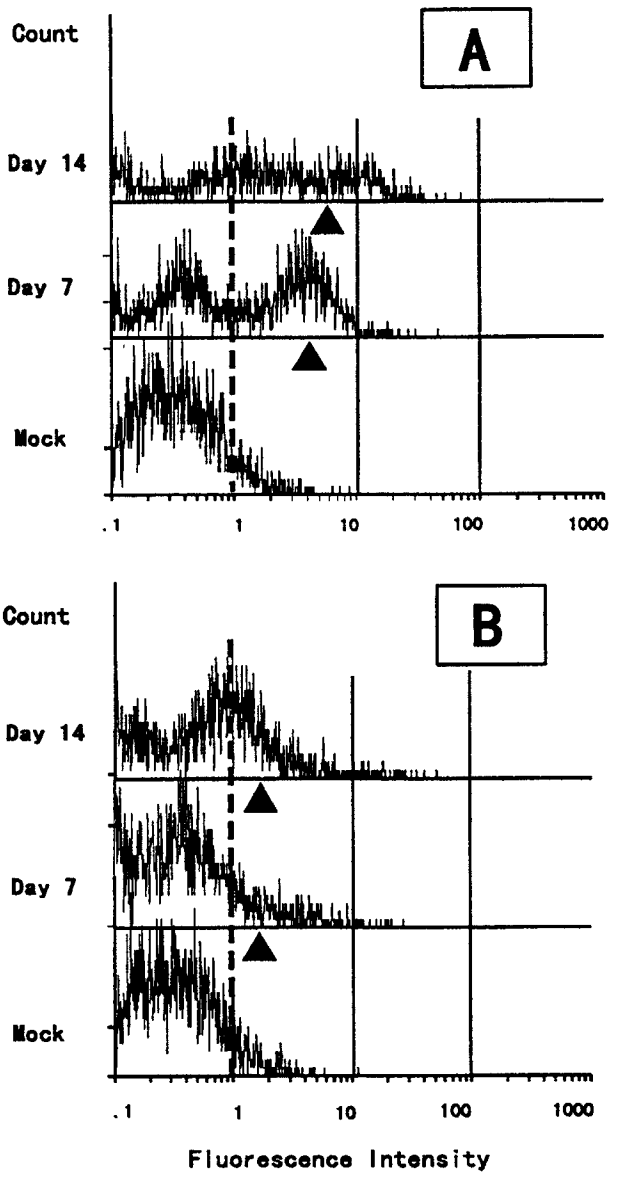

Fig. 1
Changes in population and fluorescence intensity of alveolar macrophages containing the *C. burnetii* antigen
Alveolar macrophages from sensitive A/J (A) and resistant C57BL/6 mice (B). Cells right to the dotted line are *C. burnetii* antigen-positive. Triangles represent points of average intensity.

NO assay. The supernatants of infected L929 cells were harvested and clarified by centrifugation. Free rickettsia in the supernatants were removed by filtration through a membrane filter of 0.22 μm pore size and were stored at -80°C. The NO concentration was determined by mixing equal volumes (100 μl) of the filtrated supernatants and Griess reagent (1% sulfanilamide, 2.5% phosphoric acid, and 0.1% naphtylethylene diamine) in 96-well microtiter plates, and A_{550} was measured (MPRA4, Toyo Soda, Tokyo, Japan). The NO concentration was calculated from a