A CATIONIC ANTIMICROBIAL PEPTIDE ENHANCES THE INFECTIVITY OF *COXIELLA BURNETII*

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Received August 8, 1995

Summary. – Purified *Coxiella burnetii* (Nine Mile, phase I) ricketssiae were exposed to a synthetic peptide ($CAP37_{20-44}$) based on the amino acid sequence of CAP37 – a 37 K human neutrophil granule-associated cationic antimicrobial protein - and their capacity to infect L929 mouse fibroblast cells was assessed during a 10-day post-exposure period. Because the parasite thrives within the acidic phagolysosome we anticipated that $CAP37_{20-44}$ would have no adverse effect on the organism. This was borne out by the experiments; however, to our surprise, treated *C. burnetii* had a much greater capacity to infect L cells than the non-treated counterpart. We speculate that the peptide exhibits opsonin-like properties, enhancing attachment of the rickettsia to the host cell surface and subsequent entry.

Key words: *Coxiella burnetii;* infectivity; antimicrobial peptide

Introduction

The Q fever agent *Coxiella burnetii* thrives within a variety of cell lines and professional phagocytes inside the acidic environment of the phagolysosome (Akporiaye *et al.*, 1983; Burton *et al.*, 1978). This obligatory intracellular agent requires an acidic milieu for activating its metabolic machinery (Hackstadt and Williams, 1981). The strategies/properties employed by *C. burnetii* to survive within this potentially hostile environment are poorly understood. It does possess several enzymes which may protect it from host cell-generated superoxide anion and hydrogen peroxide: superoxide dismutase and catalase, respectively (Akporiaye and Baca, 1983). During phagocytosis of the parasite human neutrophils do not exhibit a metabolic burst with concomitant production of superoxide anion (Akporiaye *et al.*, 1990; Ferenčík *et al.*, 1984, 1985). An explanation for this inhibition may be the acid phosphatase recently discovered in the parasite's periplasmic space (Baca *et al.*, 1993). The phosphatase apparently impairs the neutrophil's capacity to generate superoxide anion (Baca *et al.*, 1993, 1994). While these three enzymes may play major roles in allowing *C. burnetii* to survive within the phagolysosome, other unidentified conditions and factors must also play a role. For example, professional phagocytes contain in their lysosomes a number of antimicrobial cationic proteins. Why *C. burnetii* is unaffected by these peptides has, to our knowledge, not been investigated. We subsequently chose to determine the fate of *C. burnetii* exposed to a synthetic antibacterial peptide ($CAP37_{20-44}$) which is based on amino acids 20-44 of CAP37, a well-characterized 37 K antimicrobial cationic protein present in the lysosomal granules of human neutrophils (Pereira *et al.*, 1993). Both $CAP37_{20-44}$ and CAP37 are active against a number of different Gram-negative and Gram-positive bacteria; both bind to bacterial lipopolysaccharide (LPS) (Pereira *et al.*, 1993; Shafer *et al.*, 1984, 1986). Maximum *in vitro* activity occurs at pH 5.0 – 5.5 (Pereira *et al.*, 1993). The mechanism of action of these molecules is unknown. As expected, the $CAP37_{20-44}$ peptide did not have a deleterious effect on *C. burnetii*; however, to our surprise, it enhanced its infectivity.

Materials and Methods

Propagation and purification of C. burnetii. Plaque-purified phase I *C. burnetii* (Nine Mile isolate) rickettsiae were obtained from M. Peacock of the Rocky Mountain Laboratory, Hamilton, Montana. They were cultivated in our laboratory in L929 mouse

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Abbreviations: LPS = lipopolysaccharide

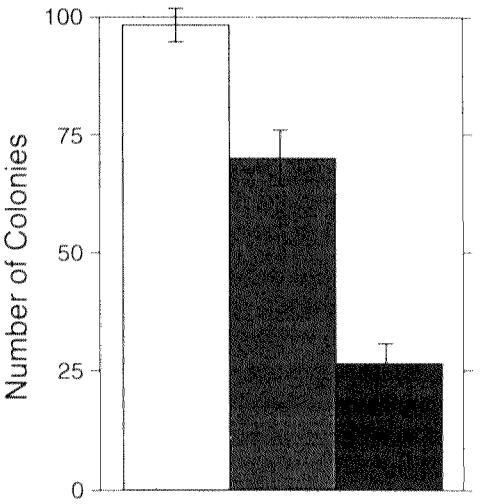

Fig. 1
Effect of $CAP37_{20-44}$ on *S. typhimurium*
Pretreatment with $CAP37_{20-44}$ (black column), control peptide (gray column) or buffer only (white column). Average (arithmetic) colony counts (and ranges) from 2 independent experiments (each performed in triplicate).

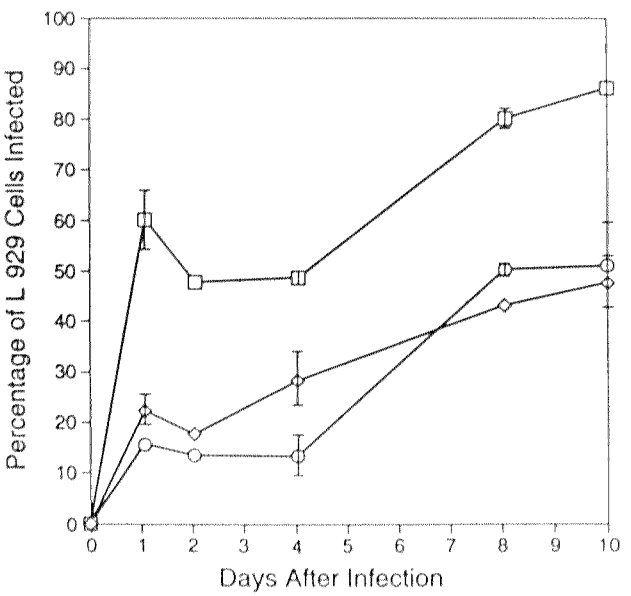

Fig. 2
Percentage of L929 cells infected with *C. burnetii* pretreated with $CPA37_{20-44}$
The infected cells contained 1 – 50 rickettsiae per cell. Pretreatment with $CAP37_{20-44}$ (□), control peptide (◇) or buffer only (○). Data are arithmetic averages (and ranges) from 2 independent experiments (each performed in duplicate).

fibroblasts in antibiotic-free Eagle's Minimum Essential Medium, spinner-modified, with 5% heat-inactivated calf serum and L-glutamine as described by Roman *et al.* (1986). The rickettsiae were purified from infected L929 cells using previously described methods (Roman *et al.*, 1986; Baca *et al.*, 1993) and enumerated using the method of Silberman and Fiset (1968) as modified by Williams *et al.* (1981). The purified rickettsiae were stored at -70 °C in 10 mmol/l Hepes buffer containing 137 mmol/l NaCl (pH 7.4).

Determination of degree of infection. The percentage of L929 cells infected and the degree of infection were determined by direct microscopic examination as described by Yeaman *et al.* (1987). Cells were deposited on glass slides with the aid of a Cytospin centrifuge (Shandon, Cheshire, England) and stained (Gimenez, 1964). A minimum of 300 cells were examined in each prepared slide to determine the percentage of the population that was infected (1 to 50 rickettsiae per cell) and heavily infected (above 50 rickettsiae per cell). Photomicrographs were made with Ektachrome film (Eastman Kodak Co., Rochester, NY).

Exposure of C. burnetii to the synthetic peptide $CAP37_{20-44}$ and assessment of effect. $CAP37_{20-44}$ and a control peptide were prepared according to previously described protocol (Pereira *et al.*, 1993). *C. burnetii* rickettsiae were exposed to the peptides using the procedures and peptide concentrations described by Pereira *et al.* (1993) in their studies to determine the effects of the peptides on a variety of bacteria, including *Salmonella typhimurium*. The antibacterial activity of $CAP37_{20-44}$ was assessed throughout the experimentation using a clinical isolate of *S. typhimurium* obtained from the New Mexico Department of Health Scientific Laboratory Division, Albuquerque NM, USA. Different clinical and laboratory strains of *S. typhimurium* were previously shown by Pereira *et al.* (1993) to be highly susceptible to $CAP37_{20-44}$. A 24-hr culture of *S. typhimurium* was transferred to fresh trypticase soy broth and incubated at 37 °C until the appropriate cell density was attained (A_{650} = 5 x 10^8 cells/ml). The bacteria were washed in ice-cold saline (0.1 ml culture in 5 ml of 0.145 mol/l NaCl) and pelleted at 2,000 x g for 10 mins at 4 °C. The pelleted cells were resuspended in 5 ml of tryptone-NaCl (0.5% (w/v) tryptone, 0.5% (w/v) NaCl) and diluted to 8 x 10^3 CFU/ml. One-tenth ml of the bacterial suspension was added to an equal volume of stock $CAP37_{20-44}$ solution (final concentration 200 µg/ml) in microtiter plates and incubated for 1 hr at 37 °C. Aliquots (0.1 ml) of the mixture were spread on trypticase soy agar plates and incubated at 37 °C for 24 hrs for subsequent enumeration of colonies.

The *C. burnetii* concentration was adjusted to 1.25 x 10^9 microorganisms/ml using P-25 buffer pH 4.5 (50 mmol/l potassium phosphate, 152.5 mmol/l potassium chloride, 15 mmol/l sodium chloride and 100 mmol/l glycine supplemented with 5 µmol/l glutamate) of Hackstadst and Williams (1981). After incubation at 37 °C for 15 mins in a microtiter plate to activate the rickettsiae, they were exposed at 37 °C for 1 hr to the $CAP37_{20-44}$ peptide at a final concentration of 200 µg/ml, a concentration shown by Pereira *et al.* (1993) to be optimal for antibacterial activity. L929 cells were then exposed to the treated *C. burnetii* at a parasite to host cell ratio of 500:1. One tenth ml of rickettsial suspension was added to a Petri dish containing 5.0 ml of L929 cells in complete growth medium (initial L929 cell concentration was 2 x 10^5 cells/ml). The cells were incubated at 37 °C (5% CO_2 atmosphere) and passaged twice a week. At each passage slides were prepared and stained to assess the degree of infection during the 10-day period.

Viability of L929 cells was assayed by the dye-exclusion method of Phillips (1973).

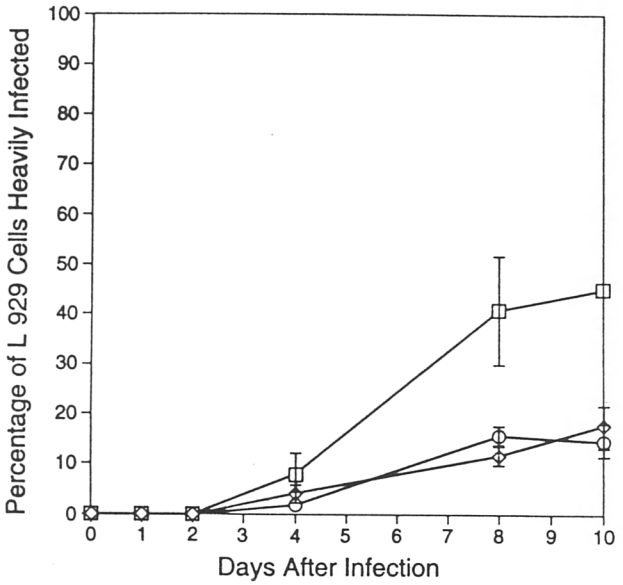

Fig. 3
Percentage of L929 cells heavily infected with *C. burnetii* pretreated with $CAP37_{20-44}$
The infected cells contained more than 50 rickettsiae per cell. Pretreatment with $CAP37_{20-44}$ (□), control peptide (◇) or buffer only (O). Data are arithmetic averages (and ranges) from 2 independent experiments (each performed in duplicate).

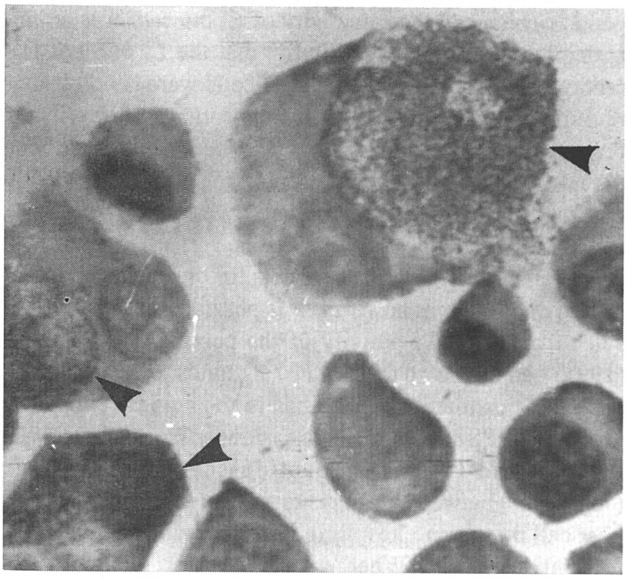

Fig. 4
Photomicrograph of L929 cells infected for 10 days with *C. burnetii* pretreated with $CAP37_{20-44}$
The cells stained by the Gimenez (1964) method. Note heavily infected cells; several contain hundreds of rickettsiae within phagolysosomes (arrowheads). Magnification 3000 x.

Results

Because *C. burnetii* is an obligate intracellular parasite, the effect of the synthetic peptide $CAP37_{20-44}$ on the purified organism was assessed by determining the capacity of the treated rickettsiae to infect L929 mouse fibroblasts.

Experiments were simultaneously conducted with *S. typhimurium* to confirm the bactericidal activity of $CAP37_{20-44}$. Treatment of *S. typhimurium* with $CAP37_{20-44}$ (200 μg/ml, the concentration used by Pereira *et al.*, 1993) caused a 73% reduction in viability, thus confirming the antibactericidal property of the peptide (Fig. 1). A control peptide, based on the amino acid sequence 1-25 of CAP37 and with the same number of amino acids (25) as $CAP37_{20-44}$ did affect, somewhat, the viability of the bacteria but to a significantly lesser degree.

Unexpectedly, instead of affecting the viability of *C. burnetii* (as assessed by its capacity to infect L929 cells) the treatment of the rickettsiae with $CAP37_{20-44}$ (200 μg/ml)) resulted in an enhanced capacity to infect L929 cells (Fig. 2). On the first day after exposure of the L929 cells to the $CAP37_{20-44}$-treated *C. burnetii*, 60% of the cells were infected with one or more rickettsiae. L929 cell populations exposed either to *C. burnetii* treated with the control peptide or exposed only to the buffer (untreated control) exhibited levels of infection after the first day of 23% and 16%, respectively. This difference was maintained for the duration of the experiment. L929 cell populations exposed to $CAP37_{20-44}$-treated rickettsiae began to exhibit heavy infection (>50 rickettsiae/cell) after the fourth day post infection (Figs. 3 and 4). By the tenth day, the proportion of heavily infected L929 cells exposed to $CAP37_{20-44}$-treated rickettsiae represented 45% of the total cell population; L929 cells exposed to control peptide-treated *C. burnetii* or buffer only (untreated controls) were in 18% and 15%, respectively, heavily infected. The viabilities of all L929 cell populations were 80% or higher (average of 90%) during the entire experimentation.

Most of these experiments were conducted using the same concentration of $CAP37_{20-44}$ as that reported to be effective against a variety of bacteria – 200 μg/ml. We also tested lower concentrations – 50 and 100 μg/ml – of $CAP37_{20-44}$. These lower concentrations of the peptide also enhanced, but to a lesser degree and in a dose-dependent manner, the infectivity of *C. burnetii*.

Discussion

The fact that *C. burnetii* multiplies within the phagolysosomes of professional phagocytes indicates that the parasite is resistant to the antibacterial cationic peptides present in lysosomes. It was anticipated that *in vitro* treatment of *C. burnetii* with the antibacterial cationic peptide $CAP37_{20-44}$ would not have a deleterious effect on the parasite. That the

peptide enhanced the infectivity of *C. burnetii* was an unanticipated result. The possibility that the *C. burnetii* microorganisms observed within the cells were dead or nonmultiplying was excluded by the appearance of heavily infected cells. Killed or inhibited *C. burnetii* would have been digested by the L929 cells. Earlier, Yeaman *et al.* (1987) reported that *C. burnetii* inhibited by antibiotics was rapidly dissolved by the host L929 cells.

The mechanism(s) that accounts for the enhanced infectivity is not known; however, it is possible that it is the result of opsonin-like activity of the peptide. Pereira *et al.* (1993) demonstrated that $CAP37_{20-44}$ binds to bacterial LPS and have postulated that it binds to the anionic phosphate groups of LPS via its cationic residues. We speculate that the cationic $CAP37_{20-44}$ peptide serves as a bridge ("opsonin") between the parasite and the negatively charged host cell membrane, resulting in an overall accelerated infection of the cell. Whether or not extracellular cationic peptides play a role *in vivo* in the entry of *C. burnetii* into host cells is unknown. It is conceivable that during the inflammatory process, and the attendant release of lysosomal contents from neutrophils, cationic peptides might subsequently coat extracellular *C. burnetii* resulting in accelerated invasion of host cells.

The effect of other cationic antimicrobial proteins from platelets and cells of the monocytic series on *C. burnetii* should also be examined. Based on the present results, one might predict that they too would enhance the infectivity of the parasite.

The surface properties of *C. burnetii* which contribute to its capacity to withstand the killing effect of the cationic peptide are unknown and should be investigated.

Acknowledgement. This work was supported by grants from the US Public Health Service to O.G.B. (AI-32492) and H.A.P. (AI-28018). H.A.P.'s work was also aided by grants from the Presbyterian Health Foundation and the American Cancer Society.

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