Cryptdin-2: a novel therapeutic agent for experimental Salmonella Typhimurium infection

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Objectives: Salmonella infections represent a major health hazard and have been responsible for a number of epidemics. In view of the emergence of multidrug-resistant Salmonella strains, there is a need for therapeutic alternatives. The purpose of this study was to evaluate the therapeutic potential of cryptdin-2 (a Paneth cell antimicrobial peptide) against Salmonella infection.

Methods: The bactericidal activity of cryptdin-2 against Salmonella enterica serovar Typhimurium NCTC74 was evaluated in vitro, ex vivo and in vivo on the basis of cfu enumeration.

Results: The MBC of cryptdin-2 for Salmonella Typhimurium was found to be 19 mg/L. The ex vivo study demonstrated significantly higher intracellular killing of the bacteria by macrophages treated with cryptdin-2 as compared with untreated macrophages. Treatment of infected mice with cryptdin-2 resulted in significant clearance of Salmonella from livers, spleens and intestines.

Conclusions: The therapeutic efficacy of cryptdin-2 suggests that it may be a promising option to combat Salmonella infections or at least may act as an adjunct to conventional antibiotics.

Keywords: Salmonella Typhimurium, antimicrobial peptides, therapeutic efficacy

Introduction

The emergence of Salmonella strains resistant to first-line antibiotics,1 as well as showing increased MICs of second-generation quinolones,2 is a serious problem limiting the possibilities for effective treatment of human Salmonella infections. It necessitates the exploitation of alternative antibacterial therapies. One such alternative is the possible therapeutic use of cationic antimicrobial peptides (AMPs). AMPs are effector molecules of the innate immune system and possess direct antibacterial and immunomodulatory properties. Earlier studies have indicated the in vitro and in vivo therapeutic effects of various cationic AMPs including human neutrophil peptides against bacterial1 and viral1 infections. The antibacterial activity of cryptdins (mouse Paneth cell defensins) has only been investigated in vitro against a few microbes,2 but the in vivo therapeutic potential is yet to be investigated. The present study was therefore planned to evaluate the in vitro, ex vivo and in vivo antimicrobial efficacy of synthetic cryptdin-2 against Salmonella enterica serovar Typhimurium NCTC74.

Materials and methods

Bacterial strain

The bacterial strain S. enterica serovar Typhimurium NCTC74 (obtained from the Central Research Institute, Kasauli, India) was used in the present study. This strain has been used in previous studies both as a virulent strain6 and as a reference strain.7

Animals

BALB/c mice (18–22 g) of either sex (4–5 weeks old) obtained from the Central Animal House, Panjab University, Chandigarh, India were housed under standard conditions with free access to feed and water ad libitum. Throughout the study, the guidelines of the Institutional Animal Ethics Committee, Panjab University, Chandigarh (India) were followed.

Synthetic cryptdin-2

A chemically synthesized peptide with the amino acid sequence LRDLVCYCRTRGCKRRERMNGTCRKGHLMYTLCCR, identical to the sequence of mouse Paneth cell cryptdin-2, was obtained from Taurus Scientific,
USA. It was suspended in 0.01% acetic acid, stored as a stock solution of 100 mg/L at −20°C and used within 3 weeks.

**Determination of the MBC of cryptdin-2 (in vitro)**

To study the in vitro effect of cryptdin-2 on the growth of Salmonella Typhimurium NCTC74, flat-bottom tubes containing bacterial cells and the peptide at different concentrations were incubated at 37°C on an orbital shaker until the mid-exponential phase (16–18 h). The MBC (at which there was >99% inhibition of growth) was calculated by monitoring the cfu at various concentrations with respect to the untreated cells.

**Intracellular killing of Salmonella Typhimurium (ex vivo)**

Mouse peritoneal macrophages were infected with Salmonella Typhimurium at a multiplicity of infection of 1:100. Extensively washed infected macrophages were treated with 12 mg/L (pre-optimized dose) cryptdin-2. After every 30, 60 and 90 min of the treatment period, treated and untreated macrophages were pelleted (2000 rpm, 10 min) and lysed with 500 μL of chilled 0.25% Triton X-100. Lysates were serially diluted and plated on MacConkey agar medium. After an incubation period of 24 h at 37°C, the number of cfu was counted.

**Therapeutic potential of cryptdin-2 against experimental Salmonella infection (in vivo)**

Mice were infected with 0.25 mL of 2·5⋅10⁷ cfu/mL Salmonella Typhimurium orally. Seven days after challenge, establishment of Salmonella infection was confirmed by the bacterial translocation in intestines, livers and spleens of infected mice. To investigate the therapeutically efficacious activity of cryptdin-2, the infected animals were randomized into three groups, with 20 mice in each treatment group (Group I and Group II) and 30 mice in the control group (Group III). Group I was subcutaneously administered cryptdin-2 at a single dose of 5 μg per mouse, whereas Group II was given two doses of 5 μg per mouse on consecutive days (10 μg/mouse). Group III (control group of 30 mice) was injected similarly with 0.1 mL of sterile saline. Five animals from each group were sacrificed at 12, 24, 36 and 48 h after therapy with 5 μg cryptdin-2, respectively, and their small intestines, livers and spleens were removed aseptically. Tissues were weighed and 10% homogenates were prepared in PBS. Serial 10-fold dilutions of each homogenate were plated on MacConkey agar medium for enumeration of cfu in different organs 48 h after treatment as compared with untreated controls (data not shown). Further, increasing the dose of cryptdin to 10 μg resulted in an ~3 log unit decrease in cfu from spleens and a 1.6 log unit decrease in cfu from small intestines 48 h after treatment (data not shown).

**Results**

Cryptdin-2 decreased the cfu of Salmonella Typhimurium in vitro in a concentration-dependent manner. When Salmonella Typhimurium cells were incubated with 5, 10, 15, 20 and 25 mg/L cryptdin-2, no visible growth was observed at concentrations >20 mg/L cryptdin-2. To evaluate the MBC of cryptdin-2, bacterial cells were incubated with 15, 16, 17, 18, 19 or 20 mg/L cryptdin-2. No cfu of Salmonella Typhimurium were observed when the cells were incubated with 19 mg/L and higher concentrations of cryptdin-2, indicating this concentration as the MBC. The MBC of cryptdin-2 was not affected in the presence of 5% serum although there was an increase in MBC (22 mg/L) in the presence of 10% and 15% serum concentrations, but no loss of activity was observed (data not shown).

Treatment of infected peritoneal macrophages with cryptdin-2 for different time periods indicated time-dependent intracellular killing of Salmonella Typhimurium. There was 41.1% and 91.1% killing after 30 and 90 min of treatment with the peptide, respectively (P<0.01). In contrast, in the untreated macrophages, the intracellular killing was found to be 31.1% and 77.7% at these times, respectively (Table 1).

A time- and dose-dependent decrease in the bacterial loads in livers, spleens and small intestines of the cryptdin-2-treated animals was observed in vivo suggesting the therapeutic activity of cryptdin-2 against Salmonella Typhimurium infection. Cryptdin-2 at a therapeutic dose of 5 μg given subcutaneously 7 days after the establishment of infection resulted in a 2.7 log unit decrease in cfu from livers with respect to control, a 2.5 log unit decrease in cfu from spleens and a 1.6 log unit decrease in cfu from small intestines 48 h after treatment (data not shown). Further, increasing the dose of cryptdin to 10 μg resulted in an ~3 log unit decrease in cfu from all three organs 48 h after treatment as compared with untreated controls (P<0.001) (Figure 1).

To further substantiate the therapeutic potential of cryptdin-2, both ex vivo and in vivo toxicity studies were carried out. It was found to exhibit very low cytotoxicity (<20%) towards macrophages even at a concentration twice that of the MBC (data not shown). In vivo toxicity tests revealed that serum levels of urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were comparable in both cryptdin-2-treated and untreated groups. Statistical analysis was done using Jandel Sigma Stat Statistical Software, version 2.0. P values <0.05 indicated statistical significance.

<table>
<thead>
<tr>
<th>Duration of intracellular incubation (min)</th>
<th>cfu (mean ± SD) (N)</th>
<th>Mean percentage killing ([N₀ − Nₙ/N₀] × 100)</th>
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<tbody>
<tr>
<td></td>
<td>untreated macrophages</td>
<td>treated macrophages</td>
</tr>
<tr>
<td></td>
<td>untreated macrophages</td>
<td>treated macrophages</td>
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<tr>
<td>0 (N₀)</td>
<td>9 × 10⁷</td>
<td>—</td>
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<tr>
<td>30</td>
<td>61 × 10⁶ ± 0.94</td>
<td>53 × 10⁶ ± 1.23</td>
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<td></td>
<td>32 × 10⁶ ± 0.76</td>
<td>77.7 ± 0.62%</td>
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<tr>
<td>60</td>
<td>48 × 10⁶ ± 0.5</td>
<td>31.1 ± 4.93%</td>
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<td>64.4 ± 2.98%</td>
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<tr>
<td>90</td>
<td>20 × 10⁶ ± 1.3</td>
<td>7.16 ± 2.98%</td>
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<td></td>
<td>4.93%*</td>
<td>3.98%*</td>
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*P<0.01, versus untreated macrophages.
and control mice (P > 0.05), which indicates no toxicity of the peptide towards the kidney and liver [see Table S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)].

**Discussion**

Various α-defensins, i.e. human neutrophil defensin 1 (HNP1), HNP2 and HNP3, have been evaluated for their broad range of antimicrobial activities, particularly due to the fact that they can be easily purified from human leucocytes. However, very little is known about the antimicrobial activities of α-defensins present in both human and mouse Paneth cells.8 The in vitro efficacy of cryptdin-2 observed in previous studies as well as in the present study prompted us to investigate its ex vivo and in vivo therapeutic efficacy against Salmonella Typhimurium.

Salmonella Typhimurium infection of macrophages is known to increase intracellular reactive oxygen intermediates (ROIs), which in turn increase the expression of other antimicrobial factors.9 Thus the enhanced killing of the Salmonella in the presence of cryptdin-2 observed in the present study might be due to the combined effect of macrophage antibacterial functions and the bactericidal activity of cryptdin-2.

As little as 5 μg of cryptdin-2 per mouse was found to clear Salmonella under in vivo conditions in the present study, compared with 19 mg/L required under in vitro conditions. Moreover, an increased clearance of bacterial loads in spleens indicated that cryptdin-2 might also play a role in clearance of bacteria at the systemic level. The in vivo toxicity tests carried out using renal and liver function tests indicated no effect of the peptide on these organs (Table S1). Our results are in concordance with previous studies carried out by Kuckelhaus et al.10

Although there have been studies regarding the in vitro role of cryptdin-2 against several pathogens, including Salmonella, this is the first report indicating the therapeutic potential of cryptdin-2 against experimental salmonellosis without any significant cytotoxic effect. Further studies are currently being carried out to explore the possibility of using cryptdin-2 as an adjunct to conventional antibiotics for the treatment of salmonellosis.

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**Transparency declarations**

None to declare.

**Supplementary data**

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**