In Vitro and In Vivo Synergistic Effects of Cryptdin 2 and Ampicillin against Salmonella[▽]

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In view of the emergence of multidrug-resistant *Salmonella* strains, there is a need for therapeutic alternatives. To reduce the dose of antibiotic required in order to decrease the associated side effects, the present study was aimed at evaluating the synergism between cryptdin 2 (a Paneth cell antimicrobial peptide) and ampicillin (Amp) against *Salmonella enterica* serovar Typhimurium. The synergy was evaluated in terms of the fractional bactericidal concentration (FBC) index, time-kill assay results (*in vitro*), macrophage functions, i.e., intracellular killing, lipid peroxidation, superoxide dismutase activity, and generation of nitrite (*ex vivo*), and decreases in CFU of salmonellae in livers, spleens, and small intestines of infected mice treated with cryptdin 2 and/or Amp (*in vivo*). *In vitro* synergism between the two agents was observed on the basis of the FBC index and time-kill assays. When the agents were used in combination, *ex vivo* studies revealed an enhanced effect on macrophage functions, particularly exhibiting a synergetic effect in terms of SOD levels. *In vivo* synergy was indicated by larger log unit decreases in all target organs of mice treated with the combination than those for the drugs used alone. These results point toward the possible use of cryptdin 2 as an adjunct to ampicillin and may help in developing alternate strategies to combat *Salmonella* infections.

Emergence of *Salmonella* strains resistant to first-line antibiotics, as well as increased MICs of quinolones, is tending to be a serious problem limiting the possibilities for effective treatment of human *Salmonella* infections (15, 33, 34). Moreover, frequent and lengthy use of antibiotics usually results in alteration of the intestinal commensal flora and leads to chronic toxicity. Recent studies have demonstrated that the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium has nine functional drug efflux pumps (26). The expression of this multidrug efflux system has been indicated to decrease cellular drug accumulation by altering cell permeability, thereby offering resistance to quinolones and some β-lactams (27). It provides an impetus to efforts to identify and exploit alternative antibacterial therapies against *Salmonella* infections.

In this context, development of cationic antimicrobial peptides (AMPs) for the treatment of salmonellosis has recently become a major area of investigation (41). Among naturally occurring AMPs, defensins form a unique family of cysteine-rich cationic polypeptides with 3 or 4 disulfide bridges (22, 39). Mouse enteric alpha-defensins called cryptdins are broad-spectrum AMPs due to their ability to kill various bacteria (8, 12, 14, 23, 28), parasites (9), and enveloped viruses (35) *in vitro*. Recently, we demonstrated that cryptdin 2 possesses a strong *in vivo* therapeutic potential against murine salmonellosis without exhibiting any toxicity as indicated by liver and kidney function tests (29). Additionally, it was found to exhibit very low cytotoxicity toward macrophages, even at a concentration twice the minimal bactericidal concentration (MBC) (29).

It has also been reported that Paneth cell cryptdins are natural pore-forming peptides and may be capable of mediating the transport of therapeutic molecules inside the target cell (19). Hence, the use of cryptdins can be perceived as a promising solution to the growing problem of resistance to conventional antibiotics in *Salmonella*, particularly by facilitating the entry of drugs into the cell. Combination therapy with antibiotics and defensins can therefore be used to increase the *in vivo* activity as well as to broaden the antimicrobial spectrum (13). Earlier, several AMPs were used in combination with conventional drugs against various bacteria, fungi, and viruses (10, 16, 21, 31, 42). However, until now, to the best of our knowledge, no information has been available on the combined activity of cryptdins and conventional anti-*Salmonella* drugs. The present study was therefore planned to evaluate the *in vitro*, *ex vivo*, and *in vivo* synergistic effects, if any, of cryptdin 2, a mouse Paneth cell alpha-defensin, in combination with ampicillin (Amp), an antibiotic conventionally used against *Salmonella*.

MATERIALS AND METHODS

Bacterial strain and growth medium. *Salmonella* Typhimurium NCTC74, procured from Central Research Institute, Kasauli, India, was used in the present study. This strain was maintained on MacConkey agar medium and has been used in earlier studies as both a virulent and a reference strain (29). Overnight cultures were harvested by centrifugation ($3,783 \times g$, 10 min), washed once with 10 mM sodium phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS to a final concentration of approximately 1×10^7 cells/ml.

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

Synthetic cryptdin 2 and ampicillin. A chemically synthesized peptide with the amino acid sequence LRDLVCYCRTRGCKRRERMNGTCRKGHLMYTL CCR, identical to the sequence of mouse Paneth cell cryptdin 2, with disulfide linkages at Cys[I]-Cys[VI], Cys[II]-Cys[IV], and Cys[III]-Cys[V], was obtained from Taurus Scientific. It was suspended in 0.01% acetic acid, stored as a stock solution at 100

mg/liter at −20°C, and used within 3 weeks. Amp powder was procured from Sigma-Aldrich.

***In vitro* activity of cryptdin 2 and Amp against *Salmonella*. (i) MBCs.** The anti-*Salmonella* activity of cryptdin 2 and Amp was monitored by a broth dilution technique. In brief, cells were grown individually in the presence of different concentrations of cryptdin 2 (5 to 20 μg/ml) and Amp (0.5 to 16 μg/ml) in specially designed flat-bottom tubes containing 3 ml of nutrient broth (5.0 g/liter peptone, 5.0 g/liter NaCl, 1.5 g/liter beef extract, 1.5 g/liter yeast extract, pH 7.4 ± 0.2), followed by the addition of an inoculum of 10^7 cells of *S.* Typhimurium. Growth was monitored during the mid-log phase by measuring the optical density at 620 nm. In addition, cell cultures from the mid-log phase were diluted appropriately in PBS and plated on MacConkey agar medium. After 16 to 18 h, the number of CFU was enumerated, and the data obtained were used to calculate the MBCs of cryptdin 2 and Amp. The MBC was defined as the concentration at which there was >99% inhibition of growth.

(ii) FBCs. A checkerboard test was performed in 96-well microtiter trays, using an 8-by-8 well configuration. Twofold serial dilutions of cryptdin 2 and Amp were prepared, with concentrations ranging from 0 to 2 times the MBC. Ten microliters of each cryptdin 2 dilution was added to the wells of a 96-well plate in a vertical orientation, and 10 μl of each Amp dilution was added in a horizontal orientation so that the plate contained various combinations of the two agents. Each well was then supplemented with 80 μl (10^7 CFU/ml) of *S.* Typhimurium, and the plate was incubated at 37°C. Wells not containing any antibacterial agent were used as positive growth controls. The fractional bactericidal concentration (FBC) was calculated by dividing the MBC of the combination of cryptdin 2 and Amp by the MBC of cryptdin 2 or Amp alone. The FBC index, obtained by adding both FBCs, was interpreted as indicating a synergistic effect when it was ≤0.5, an additive or indifferent effect when it was >0.5 and ≤2.0, and an antagonistic effect when it was >2.0 (42).

(iii) Time-kill assay. To determine the bactericidal action of cryptdin 2 and Amp, separately and in combination, *S.* Typhimurium was exposed to one of the antimicrobial agents or to both simultaneously, and the viable count was monitored. Cryptdin 2 (19 μg/ml) and Amp (4.0 μg/ml), alone and in combination (cryptdin 2 at 5.0 μg/ml plus Amp at 0.5 μg/ml), were added to nutrient broth (5.0 g/liter peptone, 5.0 g/liter NaCl, 1.5 g/liter beef extract, 1.5 g/liter yeast extract, pH 7.4 ± 0.2) containing 10^7 CFU of *S.* Typhimurium and then incubated at 37°C. One-hundred-microliter aliquots were withdrawn at 0, 30, 60, 90, and 120 min and spread plated on MacConkey agar plates. The plates were then incubated at 37°C for 24 h for enumeration of CFU.

***Ex vivo* effect of cryptdin 2 and Amp.** Murine peritoneal macrophages were isolated by a method previously described by us (29). Briefly, peritoneal macrophages were isolated by intraperitoneal elicitation of mice with 1 ml of 3% thioglycolate medium. Two days later, mouse peritoneal cavity fluids were flushed with 8 to 10 ml chilled RPMI 1640 containing 10% fetal calf serum (FCS) and then centrifuged, and macrophages were resuspended in cold RPMI 1640. Cell viability was checked by the trypan blue exclusion method.

Intracellular killing of *S.* Typhimurium. Mouse peritoneal macrophages were infected with *S.* Typhimurium at a multiplicity of infection (MOI) of 1:100 for 90 min. Infected macrophages were washed three times in RPMI 1640 and then treated with cryptdin 2 (19 μg/ml) and Amp (4 μg/ml), separately and in combination at reduced concentrations (cryptdin 2 at 5.0 μg/ml plus Amp at 0.5 μg/ml). After every 30, 60, and 90 min, treated and untreated macrophages were pelleted (2,000 rpm, 10 min) and lysed with 500 μl of 0.25% Triton X-100. Lysates were serially diluted and plated on MacConkey agar medium. After incubation for 24 h at 37°C, the number of CFU was counted. The mean percent intracellular killing was calculated as described earlier (29). Values are expressed as means ± standard deviations (SD) for three independent experiments. Statistical analysis was done by Student's *t* test, and *P* values of <0.05 were considered statistically significant.

Interaction of macrophages with *S.* Typhimurium. Since *Salmonella* is an intracellular pathogen, *Salmonella*-macrophage interactions play a central role in its pathogenesis. *S.* Typhimurium encounters oxidative stress within the macrophage milieu, mounted in the form of the respiratory burst. The respiratory burst that is concomitant with phagocytosis produces a number of toxic by-products. Therefore, to assess the effects on the extent of lipid peroxidation (LPO) and on superoxide dismutase (SOD) activity and to measure nitric oxide generation, murine peritoneal macrophages were incubated with *Salmonella* at an MOI of 1:100 in the presence and absence of cryptdin (19 μg/ml) and Amp (4 μg/ml), alone and in combination at reduced concentrations (cryptdin 2 at 5.0 μg/ml plus Amp at 0.5 μg/ml), in a six-well tissue culture plate at 37°C in a CO$_2$ incubator for 18 h. After incubation, lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF]) was added (1:1 ratio) to all the wells, and the plate was further incubated at 4°C for

20 min. Reaction mixtures from each well were centrifuged (2,000 rpm, 15 min), and supernatants thus obtained were used to study the following.

(i) Extent of LPO. Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids (PUFAs). The peroxidation of PUFAs is a feature of many types of cell injuries in which free radical intermediates are produced in excess. Lipid peroxidation can cause changes in membrane fluidity and permeability and can increase the rate of protein degradation, which eventually leads to cell lysis. A measure of LPO products, such as malondialdehyde (MDA), is an indication of the extent of damage. Quantitative measurement of lipid peroxidation in the culture supernatants of macrophages was performed as described by Wills (40). The results are expressed as nanomoles of MDA per milligram of protein, using the molar extinction coefficient of the chromophore (1.56×10^5 M^{-1} cm^{-1}). The protein content of the samples was estimated by the method described by Lowry et al. (20).

(ii) Estimation of nitrite concentration. Nitric oxide is an important signaling molecule regulating a diverse range of physiological processes. Nitrite levels were measured by a slight modification of the method of Green et al. (11), as described earlier (5), and served as an indicator of nitric oxide production. One-hundred-microliter aliquots of sample were mixed with 400 μl of distilled water and 500 μl of Griess reagent. The reaction mixture was incubated at room temperature for 10 min in the dark, and the optical density was measured at 546 nm. Nitrite was quantified using a standard graph for sodium nitrite. Nitrite levels in the cell-free supernatants of infected macrophages (treated with the agents) were compared with those of control (uninfected) and infected (untreated) macrophages.

(iii) Estimation of SOD activity. Host cells are protected from oxygen-derived radical injury by naturally occurring free radical scavengers and antioxidant pathways, including superoxide dismutase (SOD). However, the antioxidant defense mechanism fails due to overproduction of free radicals, decreased activities of scavenging enzymes, or both, causing lipid peroxidation. Therefore, SOD activity was assayed according to the method of Kono (17). The reaction was initiated by the addition of 0.5 ml of hydroxylamine hydrochloride to a reaction mixture containing 2 ml of nitroblue tetrazolium (NBT) and 0.1 ml of culture supernatant. The change in absorbance was measured spectrophotometrically at 560 nm. SOD activity is expressed as units of SOD per milligram of protein, where 1 unit of activity is defined as the amount of SOD required to inhibit the rate of reduction by NBT by 50%.

***In vivo* effect of cryptdin 2 in conjunction with Amp against murine salmonellosis.** Mice were infected with 10^7 CFU of *Salmonella* Typhimurium orally. Seven days after the challenge, establishment of *Salmonella* infection was confirmed by bacterial translocation into the intestines, livers, and spleens of the infected mice. At 7 days postinfection, mice were randomized into 11 groups of five mice each and treated with cryptdin 2 and ampicillin. Cryptdin 2 was injected subcutaneously (s.c.) at a dose of 5 μg/mouse, while Amp was administered s.c. at 16, 32, and 64 mg/kg of body weight, individually and in combination. In addition to these doses, lower concentrations of both the agents in combination were also tested.

Mice in group I were injected s.c. with 0.1 ml sterile saline and served as the control (infected) group. Group II mice were administered 16 mg/kg body weight of ampicillin s.c. Group III mice were administered Amp s.c. at 32 mg/kg body weight. Group IV mice received 64 mg/kg body weight of Amp s.c. Group V mice were administered cryptdin 2 s.c. at a single dose of 5 μg/mouse. Group VI mice were coadministered cryptdin 2 (5 μg/mouse; s.c.) and Amp (16 mg/kg body weight; s.c.). Group VII mice were coadministered cryptdin 2 (5 μg/mouse; s.c.) and Amp (32 mg/kg body weight; s.c.). Group VIII mice were coadministered cryptdin 2 (5 μg/mouse; s.c.) and Amp (64 mg/kg body weight; s.c.). Group IX mice were coadministered cryptdin 2 (2.5 μg/mouse; s.c.) and Amp (8 mg/kg body weight; s.c.). Group X mice were coadministered cryptdin 2 (2.5 μg/mouse; s.c.) and Amp (16 mg/kg body weight; s.c.). Group XI mice were coadministered cryptdin 2 (2.5 μg/mouse; s.c.) and Amp (32 mg/kg body weight; s.c.). At 48 h posttherapy, mice were sacrificed and their livers, small intestines, 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 groups.

Statistical analysis. Data are expressed as means ± standard deviations for three to five independent experiments. Statistical analysis was done by Student's unpaired *t* test and one-way analysis of variance (ANOVA), followed by pairwise comparison procedures (Tukey test), using Jandel Sigma Stat statistical software, version 2.0. In all cases, statistical significance was defined as having a *P* value of <0.05.
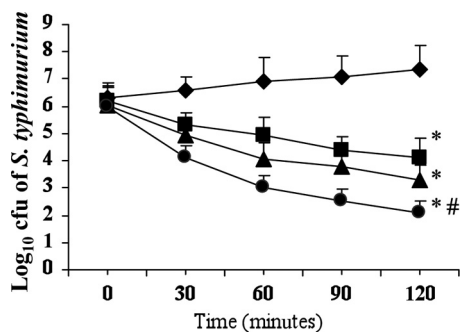
FIG. 1. $\log_{10}$ CFU of *Salmonella* Typhimurium NCTC74 at various time intervals in the presence of ampicillin alone at 4 μg/ml (■), cryptdin 2 alone at 19 μg/ml (▲), and both drugs in combination, i.e., Amp at 0.5 μg/ml plus cryptdin 2 at 5 μg/ml (●). Values are expressed as means ± SD for three independent experiments. *, $P < 0.01$ versus $\log_{10}$ CFU of *Salmonella* after 120 min in the absence of any antibacterial agent (control; ◆); #, $P < 0.05$ versus $\log_{10}$ CFU of *Salmonella* after 120 min in the presence of Amp (4 μg/ml) (■).
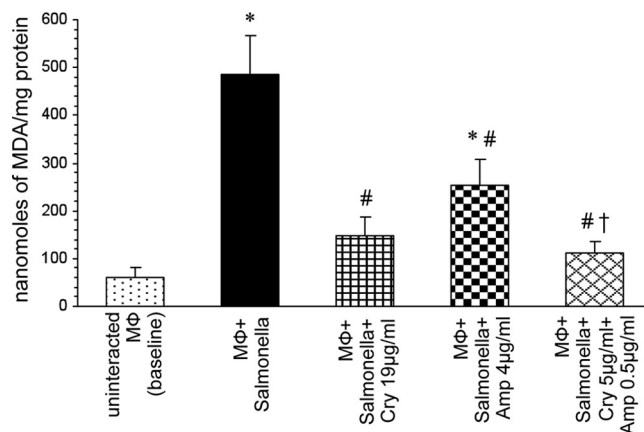

FIG. 2. Effect on MDA levels of macrophages (MΦ) incubated with *S.* Typhimurium in the presence of Amp and cryptdin (Cry) alone and in combination. Values are expressed as means ± SD for four independent experiments. Statistical analysis was done using one-way ANOVA. *, $P < 0.01$ versus uninfected macrophages (baseline); #, $P < 0.05$ versus macrophages incubated with *Salmonella*; †, $P < 0.05$ versus macrophages incubated with *Salmonella* in the presence of Amp.

RESULTS

***In vitro* activity of cryptdin 2 and Amp against *Salmonella*. (i) Minimum bactericidal concentrations.** Cryptdin 2 and Amp decreased the CFU of *Salmonella* Typhimurium *in vitro* in a concentration-dependent manner. When *Salmonella* Typhimurium cells were incubated with 0.5, 1, 1.5, 2, 4, 8, and 16 μg/ml of Amp, no visible growth was observed at 4 μg/ml (10.77 μmol/liter), indicating this concentration as the MBC of Amp against *S.* Typhimurium. Similarly, the MBC of cryptdin 2 against *Salmonella* was evaluated to be 19 μg/ml (4.47 μmol/liter), as reported earlier (29).

(ii) Fractional bactericidal concentrations. The combination of cryptdin 2 and Amp was found to be highly effective *in vitro*, as evidenced by the reduced MBCs of 5 μg/ml and 0.5 μg/ml for cryptdin 2 and Amp, respectively. The FBC index was calculated to be 0.388, which indicated *in vitro* synergy between the two agents.

(iii) Time-kill assay. After 120 min, cryptdin 2 (19 μg/ml) and Amp (4 μg/ml) administered separately gave significant decreases ($P < 0.01$), of 4.11 and 3.26 log units, respectively, compared to the control (120 min). However, when they were used in combination (cryptdin 2 at 5 μg/ml and Amp at 0.5 μg/ml), a decrease of 5.32 log units was observed after 120 min ($P < 0.05$), indicating an *in vitro* synergetic effect (Fig. 1).

Activities of cryptdin 2 and Amp against intracellular *Salmonella*. The mean percent intracellular killing in untreated macrophages was found to be 17.1% ± 3.4%, 27.6% ± 3.67%, and 43.4% ± 5.2% at 30, 60, and 90 min, respectively. On the other hand, the mean percent intracellular killing in the presence of cryptdin 2 (19 μg/ml) alone at 30, 60, and 90 min was 31.5% ± 4.98% ($P < 0.05$), 46% ± 5.16% ($P < 0.05$), and 61.8% ± 8.57%, respectively, compared to that in untreated cells. Similarly, when infected macrophages were treated with Amp (4 μg/ml) alone, the mean percent intracellular killing was 30.2% ± 4.23% ($P < 0.05$), 40.7% ± 3.95% ($P < 0.05$), and 59.2% ± 6.17% at 30, 60, and 90 min, respectively. A higher percentage of intracellular killing was observed when infected macrophages were treated with cryptdin 2 (5 μg/ml) in conjunction with Amp (0.5 μg/ml) at lower doses, indicating *ex*

vivo synergy. In this case, the mean percent killing was found to be 43.4% ± 5.5% ($P < 0.05$), 57.8% ± 6.09% ($P < 0.01$), and 82.8% ± 10.05% ($P < 0.001$) at 30, 60, and 90 min, respectively. These results indicate that both cryptdin 2 and Amp might act in conjunction with macrophage antibacterial effectors to lead to enhanced killing of intracellular salmonellae.

Estimation of lipid peroxidation levels. Significant decreases in the levels of MDA were observed when macrophages were incubated with *S.* Typhimurium in the presence of cryptdin 2 ($P < 0.01$) or Amp ($P < 0.05$) alone compared to those in macrophages incubated with *Salmonella* in the absence of any antimicrobial agent. However, a much larger reduction in MDA levels was observed ($P < 0.01$) when macrophages were incubated in the presence of both cryptdin 2 and Amp (Fig. 2).

Estimation of nitrite levels. A significant decrease ($P < 0.05$) in the level of nitrite produced by macrophages was observed in the presence of cryptdin 2 compared to that in macrophages incubated with *Salmonella* in the absence of any antimicrobial agent. Similarly, nitrite levels of infected macrophages were significantly reduced in the presence of Amp also, but to a lesser extent ($P < 0.05$) (Fig. 3). However, a more pronounced effect ($P < 0.05$) was observed when macrophages were incubated with *Salmonella* in the presence of both cryptdin 2 and Amp (Fig. 3).

Estimation of SOD levels. Interaction of macrophages with *Salmonella* induced a significant decrease in the SOD activity in the culture supernatant of macrophages ($P < 0.01$). No significant change in SOD activity was observed in the presence of cryptdin 2 or Amp alone compared to that in normally interacting macrophages in the absence of any antimicrobial agent. However, supplementation of the reaction mixture with both cryptdin 2 and Amp significantly restored the SOD activity ($P < 0.05$) (Fig. 4).

Therapeutic potential and synergetic efficacy of cryptdin 2 and Amp against experimental salmonellosis. The therapeutic efficacy of cryptdin 2 and Amp alone and in conjunction was
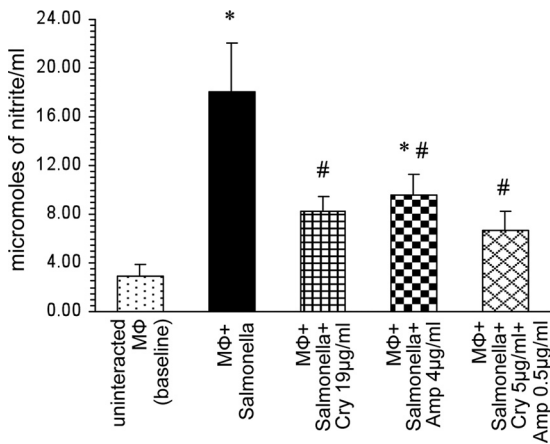
FIG. 3. Effect on nitrite levels of macrophages (MΦ) incubated with *S.* Typhimurium in the presence of Amp and cryptdin (Cry) alone and in combination. Values are expressed as means ± SD for four independent experiments. Statistical analysis was done using one-way ANOVA. *, $P < 0.05$ versus uninfected macrophages (baseline); #, $P < 0.05$ versus macrophages incubated with *Salmonella*.

FIG. 4. Effect on activity of superoxide dismutase of macrophages (MΦ) incubated with *S.* Typhimurium in the presence of Amp and cryptdin (Cry) alone and in combination. Values are expressed as means ± SD for four independent experiments. Statistical analysis was done using one-way ANOVA. *, $P < 0.05$ versus uninfected macrophages (baseline); #, $P < 0.05$ versus macrophages incubated with *Salmonella*.

investigated in terms of reduction in the number of salmonellae in different target organs of mice infected with *Salmonella* Typhimurium after 48 h of chemotherapy. In the livers of treated mice, log unit decreases in bacterial loads were 1.1, 2.2, and 3.23 for 16, 32, and 64 mg Amp/kg body weight, respectively, while a 1.96-log-unit decrease was observed after treatment with cryptdin 2. Coadministration of 5 μg of cryptdin 2 with 16, 32, and 64 mg Amp/kg body weight gave higher log unit decreases of 3, 3.79, and 4.52, respectively (Fig. 5A). In spleens of mice, log unit decreases in bacterial loads were observed to be 1.86, 2.98, 3.57, and 2.59 after treatment with 16, 32, and 64 mg Amp/kg body weight and with cryptdin 2 (5 μg), respectively. In this case also, the adjunct therapy of 5 μg cryptdin 2 with 16, 32, and 64 mg Amp/kg body weight was found to be more effective, as higher log unit decreases of 3.4, 3.61, and 3.98, respectively, were observed (Fig. 5B). In the small intestines, cryptdin 2 (5 μg/mouse) alone gave a decrease in bacterial loads of 1.67 log units compared to untreated controls. There were log unit decreases of 1.43, 1.92, and 3.25 in the number of salmonellae in small intestines of mice treated with 16, 32, and 64 mg Amp/kg body weight, respectively (Fig. 5C). However, when 5 μg of cryptdin 2 was used in combination with 16, 32, and 64 mg Amp/kg body weight, the log unit decreases in intestinal bacterial loads were found to be 2.47, 3.62, and 4.58 units, respectively. Interestingly, when the mice were treated with half of each Amp dose in conjunction with half the dose of cryptdin 2 (2.5 μg/mouse), the log unit decreases in the numbers of salmonellae in all target organs were observed to be comparable to the decreases observed when both agents were used together at 2-fold higher concentrations.

DISCUSSION

A strategy to overcome the problem of emerging drug resistance in *Salmonella* strains is to avoid overuse and misuse of antibiotics (1, 24). In this regard, the use of cationic antimicrobial peptides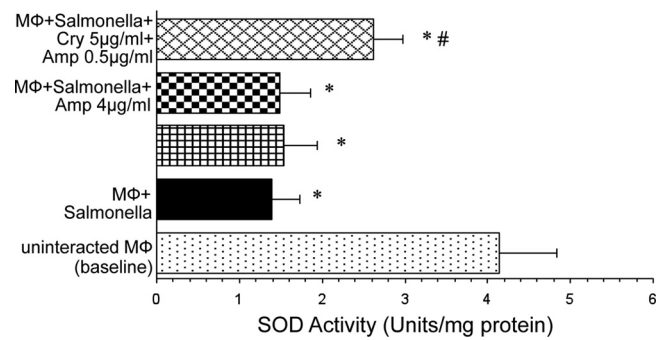 in conjunction with conventional antibiotics might prove useful, as this would reduce the dose of antibiotics required to treat the disease, thereby decreasing the associated side effects. In light of these facts, the present study aimed at evaluating the synergetic effect of ampicillin in conjunction with cryptdin 2 against *Salmonella* Typhimurium.

In vitro synergy was evidenced from the FBC index and the time-kill assay, as a larger antibacterial effect was exhibited when the two agents were used against *Salmonella* in combination at concentrations much lower than their individual MBCs. This can be attributed to an increased permeability of the *Salmonella* cell membrane created by cryptdin 2, thereby leading to an increased diffusion of Amp into the intracellular targets. These observations are consistent with previous studies pertaining to the synergetic actions of α-helical peptide p18, reproductive tract beta-defensins, HNP-1, magainin, cathelicidins, and several other novel cationic peptides with commonly used antibiotics against *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (7, 16, 32, 36, 37).

Salmonella has been reported to establish a niche within macrophages where it replicates and causes systemic disease. The dynamic interplay between host responses and bacterial virulence mechanisms determines the outcome of the infection. At this stage, antibacterial agents may directly or indirectly modulate the natural phagocyte-bacterium interactions (18). Keeping this in view, the *ex vivo* effect of cryptdin 2 and Amp on macrophage functions was evaluated in terms of intracellular killing, extent of lipid peroxidation, superoxide dismutase activity, and generation of reactive nitrogen intermediates (RNIs).

Treatment of infected macrophages with cryptdin 2 and Amp individually exhibited more killing of intracellular salmonellae than that by untreated macrophages. Interestingly, the killing effect was found to be more pronounced when both agents were used in combination to treat intracellular salmonellae. These results are in concordance with earlier reports wherein it was proposed that ampicillin kills intracellular salmonellae within mouse macrophages owing to its internalization by cells through pinocytosis (6, 18). The fact that alphadefensins may enhance phagocytosis by interacting directly
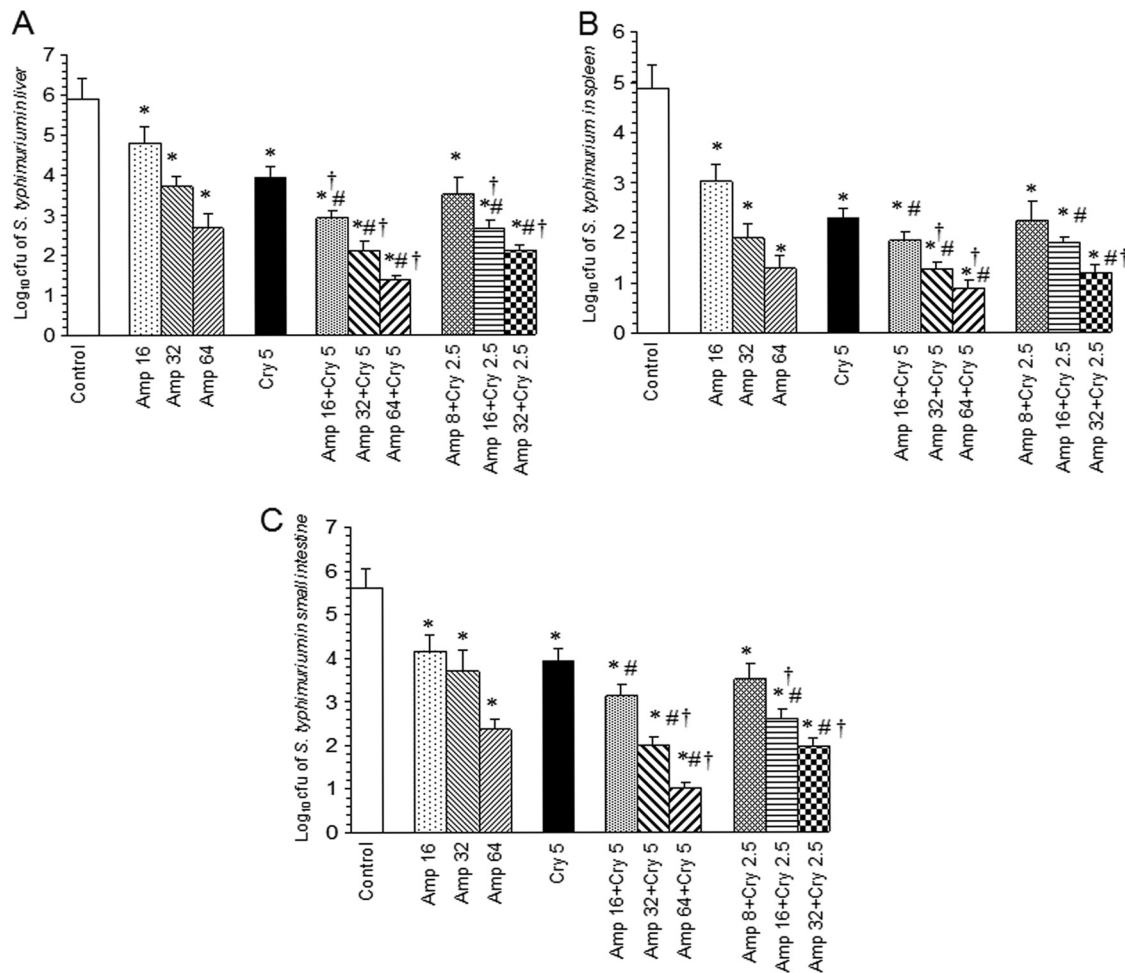
FIG. 5. Log$_{10}$ CFU of *S.* Typhimurium in livers (A), spleens (B), and small intestines (C) of infected mice after 48 h of therapy with cryptdin 2 (Cry) and ampicillin (Amp), separately and in combination. Values are expressed as means ± SD for two independent experiments. Statistical analysis was done using one-way ANOVA. ∗, $P < 0.05$ versus log$_{10}$ CFU of *S.* Typhimurium in untreated mice (control); #, $P < 0.05$ versus log$_{10}$ CFU of *S.* Typhimurium in mice treated with Amp (16 mg/kg body weight); †, $P < 0.05$ versus log$_{10}$ CFU of *S.* Typhimurium in mice treated with cryptdin (5 µg/mouse).

with the pathogen may account for the higher killing level observed when macrophages were treated with cryptdin 2 (29). In support of our findings, defensins have been reported to enable macrophages to inhibit intracellular proliferation of *Listeria* (2).

There is evidence that *Salmonella* infection results in excessive production of reactive oxygen species, thereby leading to lipid peroxidation and finally to tissue damage (25, 30). Interaction of macrophages with *Salmonella* in the presence of cryptdin 2 in conjunction with Amp decreased the extent of LPO and restored the SOD level. In particular, the SOD activity appeared to be enhanced synergistically in the presence of the combination. These results indicate that these agents might have decreased LPO by scavenging the free radicals and by upregulating the antioxidant activity, thus counteracting the oxidative stress. In agreement with our findings, it has been reported that antibacterial agents, including defensins, may directly scavenge the oxidants produced as a result of phagocyte-bacterium interaction (18). It is also possible that cryptdin 2 might contribute to inhibition of quorum sensing (in terms of

bacterial density due to direct killing), thereby rendering the number of surviving salmonellae insufficient to activate macrophages.

Nitric oxide (NO) is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes (25). However, excessive amounts of NO are potentially toxic and have been implicated in numerous pathological situations and chronic inflammation (3). RNIs such as nitrites are known to be the end products of oxidative metabolism of labile nitric oxide, and their quantification is regarded as an indicator of NO generation (5). A significantly increased level of nitrite in macrophages infected with *Salmonella* might combine with superoxide anions to form peroxynitrites, which further stimulate the production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), contributing to tissue injury. However, when macrophages were infected with *Salmonella* in the presence of cryptdin 2 alone or in combination with ampicillin, significant decreases in nitrite levels were observed. It might be possible that these agents neutralize the *Salmonella*-induced release of nitric oxide in macrophages, as

reported earlier (43). Additionally, the lower levels of nitrite observed in the macrophages treated with cryptdin 2 might be attributed to the anti-inflammatory activity of cryptdin, as has been reported for various other AMPs (4, 12).

The results of *in vivo* studies also indicated synergism between Amp and cryptdin 2, as more clearance of salmonellae from all target organs was observed when both agents were used in combination than the bactericidal effect observed when the agents were used alone at the same doses. These results are in concordance with earlier reports suggesting a synergetic effect of antimicrobial peptides with β-lactam antibiotics (16, 36). The most interesting finding of our study was that the addition of cryptdin 2 not only potentiated the effectiveness of ampicillin but also reduced the therapeutic dosage of both agents to half (from 5 μg to 2.5 μg per mouse of cryptdin 2 and from 16, 32, and 64 mg/kg to 8, 16, and 32 mg/kg of Amp) while maintaining the increased therapeutic efficacy, compared with that of the drugs alone, against experimental salmonellosis. In the present study, a significantly lower concentration of cryptdin 2 (5 μg per mouse) was found to clear *Salmonella* infection *in vivo* than that required for *in vitro* anti-*Salmonella* activity (19 μg/ml), in concordance with our earlier report (29). The therapeutic efficacy of cryptdin 2 might be attributed to the property of antimicrobial-immunomodulatory duality exhibited by host defense peptides. Earlier, various AMPs were also shown to possess a broad range of immunomodulatory properties, including chemoattraction of immune cells, induction of chemokines, and resolution of infections (4, 12, 14, 38). Thus, it can be hypothesized that cryptdin 2 may act as an antibacterial as well as immunostimulatory molecule *in vivo* in *Salmonella*-infected mice, where it is recognized as an effector molecule of innate immunity. The implication of the present study is that cryptdin 2 can be used as a possible adjunct to ampicillin for superior treatment of salmonellosis. These observations seem to be significant, as reducing the therapeutic concentrations of antibiotics may be a valuable strategy for avoiding the development of emerging antibiotic resistance. In addition to the findings of the present study, cryptdin 2 has also been observed to possess antibacterial activity against other Gram-negative organisms, such as *Yersinia enterocolitica* (MBC, 21 μg/ml) and *E. coli* (MBC, 10 μg/ml), as well as against *Staphylococcus aureus* (MBC, 12 μg/ml) and *Entamoeba histolytica* (minimum amoebicidal concentration, 4 μg/ml) (unpublished data). In light of these data, the peptide seems to be a promising broad-spectrum antimicrobial agent.

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