

NOTES

Salmonella Infection Increases Porcine Antibacterial Peptide Concentrations in Serum†

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Received 9 April 1997/Accepted 28 July 1997

PR-39 is a multifunctional neutrophil peptide involved in host defense and inflammation. To investigate the involvement of PR-39 in a *Salmonella choleraesuis* infection, a PR-39 enzyme immunoassay was developed. The concentrations of PR-39 in serum were 13.6 ± 1.9 ng/ml before challenge and increased ($P < 0.01$) threefold by 10 to 14 days postinfection. Peripheral blood neutrophil counts paralleled the changes in the concentrations of PR-39 in serum, both returned to basal values by 4 weeks postinfection. These findings suggest that the concentrations of serum PR-39 reflect the involvement of this antibacterial peptide in the host's response to an *S. choleraesuis* infection.

Neutrophils are major cellular components in inflammatory responses and play a central role in host defense against bacterial infections and diseases through oxygen-dependent and -independent microbicidal mechanisms (3, 9). The oxygen-independent mechanisms include actions of antimicrobial peptides that are synthesized in the bone marrow and stored as propeptides in neutrophil granules (3, 12). Recently, a group of these peptides was classified as members of a family called cathelicidins, because they share the highly conserved prosequence cathelin (22, 23, 27). Mature cathelicidins, which are capable of microbicidal activities, are cleaved from the proforms by endogenous elastase upon neutrophil activation and degranulation (14, 16, 26).

A porcine cathelicidin, the proline-arginine-rich 39-amino-acid peptide PR-39, has been shown to possess many functional properties, including antibacterial activity (1, 19), the ability to induce syndecan expression (4), the ability to inhibit phagocyte NADPH oxidase (20), and neutrophil chemoattractant properties (8). Although Shiomi et al. (21) have reported that levels of human defensins are increased in individuals undergoing granulocyte-macrophage colony-stimulating-factor treatment and in patients with bacterial infections (21), few studies have addressed the in vivo kinetics of antibacterial peptides in animals with bacterial infections. Here we report that concentrations of PR-39 in serum are increased in pigs challenged experimentally with *Salmonella choleraesuis*, demonstrating the pathophysiological involvement of this multifunctional peptide in an in vivo bacterial infection.

To measure the concentrations of PR-39 in biological fluids, an enzyme-linked immunosorbent assay (ELISA) was developed. Antibodies to PR-39 were generated with a synthetic

peptide that was synthesized by the Biotechnology Core Facility at Kansas State University as previously described (20), conjugated to keyhole limpet hemocyanin (Pierce, Rockford, Ill.), emulsified in adjuvant (RIBI Immunochem Research, Hamilton, Mont.), and injected into BALB/c mice and goats. Monoclonal antibodies (MAb) to PR-39 were derived from a fusion of immunized mouse splenocytes with P3X63Ag8.653 myeloma cells (American Type Culture Collection, Rockville, Md.) as described by Harlow and Lane (6). Twelve stable hybridoma clones were obtained after three limiting dilutions. One hybridoma produced immunoglobulin G1(K) (IgG1[k]) antibodies; all of the rest produced IgM(K) antibodies as determined by an ELISA subtyping kit (Bio-Rad, Richmond, Calif.). The IgG1-producing hybridoma was used to generate ascites fluid from which MAb were purified by affinity chromatography with recombinant protein G (Pierce). Polyclonal antibodies to PR-39 were produced in goats, purified after the fourth immunization on a protein G column, and then labeled with biotin-hydrazide (Pierce).

Synthetic PR-39 and several of its C-terminal, N-terminal, and central fragments (20) were used to study the epitope specificities of the MAb and polyclonal antibodies. The MAb recognized only the full-length PR-39; however, goat polyclonal antibodies reacted not only with PR-39 but also with its N- and C-terminal portions (data not shown), suggesting that PR-39 has several antigenic epitopes.

To study the interaction of these antibodies with native PR-39, neutrophil granule extracts (0.5×10^6 cell equivalents/lane) and synthetic PR-39 (0.5 μ g/lane) were separated by acid-urea polyacrylamide gel electrophoresis as previously described (18) and then electroblotted onto polyvinylidene difluoride membranes (Micro Separations, Westboro, Mass.) at 200 mA for 10 min in 0.7% acetic acid. To detect the peptides, the membranes were first blocked for 2 h at 22°C with 1% sodium caseinate in 10 mM phosphate-buffered saline (PBS)-0.1% Tween 20 (pH 7.4), followed by an incubation with mouse anti-PR-39 MAb (1 μ g/ml), goat anti-PR-39 polyclonal antibody (10 μ g/ml), or rabbit anticathelin antiserum (1:5,000; a

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† Contribution no. 97-377-J of the Kansas Agricultural Experiment Station.

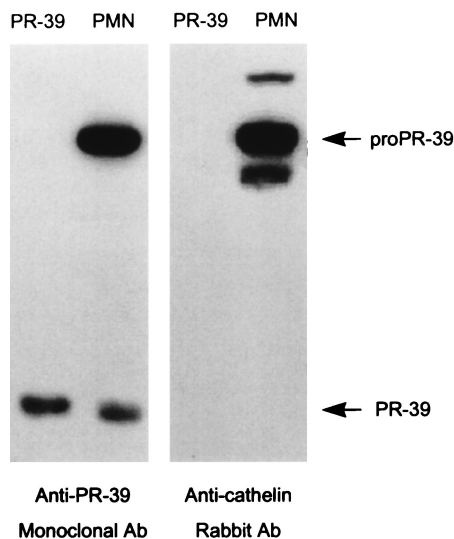


FIG. 1. Western blot analysis of synthetic and native PR-39. Synthetic PR-39 (0.5 $\mu\text{g}/\text{lane}$) and polymorphonuclear neutrophil (PMN) granule extracts (0.5 $\times 10^6$ cell equivalents/lane) were subjected to acid-urea-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The blot was developed with mouse anti-PR-39 MAb (1 $\mu\text{g}/\text{ml}$) or rabbit anticathelin antiserum (1:5,000). A chemiluminescent peroxidase substrate (SuperSignal; Pierce) was used to visualize antibody-specific binding.

kind gift from Tomas Ganz, University of California, Los Angeles) for 2 h at 22°C. After treatment for 1 h with peroxidase-labeled secondary antibodies (1:40,000; Sigma, St. Louis, Mo.), the membrane was exposed to a chemiluminescent peroxidase substrate (Pierce) to visualize specific antibody binding. As shown in Fig. 1, the MAb recognized both the mature PR-39 and the proform (cathelin-containing PR-39) in porcine neutrophil lysates. The rabbit anticathelin antibody revealed three major bands in the neutrophil lysates (Fig. 1); one was the proform of PR-39, and the other two were presumably the proforms of protegrins and prophenins, two other porcine cathelicidins (7, 10). Goat polyclonal antibodies also recognized the native mature peptide and its precursor (data not shown).

An ELISA was designed with the anti-PR-39 MAb as a capture antibody and biotinylated goat polyclonal antibody as a detecting antibody. As shown previously for human defensins (15), a cationic detergent, centrionium bromide (CETAB; Sigma), was found to be essential to obviate nonspecific binding of cationic PR-39 to microplate surfaces and the possible influence of serum components. Briefly, 96-well microplates (Corning Costar, Cambridge, Mass.) were coated overnight at 4°C with 100 μl of MAb (10 $\mu\text{g}/\text{ml}$) in 0.1 M carbonate buffer, pH 9.5. After being washed twice with 10 mM PBS-0.05% Tween 20 (PBST), the plates were blocked for 2 h at 22°C with 1% bovine serum albumin (BSA) in PBST. Synthetic PR-39 standards (0.625 to 40 ng/ml) and unknown samples were diluted with 1% BSA in PBS containing 0.01% CETAB and added to wells; then the plates were incubated at 22°C for 2 h with shaking. After four washes, biotinylated goat polyclonal antibodies (15 $\mu\text{g}/\text{ml}$ in 1% BSA-PBS with 0.01% CETAB) were added to each well and the plates were incubated for 2 h at 22°C with shaking. The plates were then incubated for 1 h with peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1:1,000 in 1% BSA-PBST, followed by color development with 2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (Kirkegaard & Perry

Laboratories, Inc.) for 30 min at 22°C. Absorbance was measured at 405 nm with a microplate reader (Cambridge Technology, Watertown, Mass.). After optimization, the sensitivity of the PR-39 ELISA was 0.5 ng/ml. The assay had a correlation coefficient of >0.99 for measurements between 0.625 and 40 ng/ml, and the intra- and interassay coefficients of variation were less than 10%.

To determine the content of PR-39 in neutrophils and its extracellular release, porcine peripheral blood neutrophils were isolated from 4- to 6-week-old pigs by density gradient centrifugation and hypotonic lysis as described previously (17). Neutrophil granule extracts were prepared by sonicating the cells twice for 30 s each time on ice in 0.2 M acetate buffer (pH 4.2) containing 5 mM EDTA, extracting them overnight at 4°C in the same buffer, and removing cell debris by centrifugation at 10,000 $\times g$ for 20 min at 4°C. The supernatants were assayed for PR-39 by the sandwich ELISA. The porcine neutrophils (10^6 cells) contained 0.44 \pm 0.09 μg of PR-39 (mean \pm standard error of the mean [SEM], three pigs). To determine if PR-39 in the neutrophils could be released by exogenous stimuli, as is the case with human and rabbit defensins (5, 11), the porcine neutrophils ($10^7/\text{ml}$) were incubated (1 h at 37°C with tumbling in Hanks' balanced salt solution, pH 7.0, containing 0.05% BSA) with medium containing either phorbol 13-myristate 12-acetate (PMA; 1 $\mu\text{g}/\text{ml}$), lipopolysaccharide (LPS; 10 $\mu\text{g}/\text{ml}$), or opsonized zymosan (OZ; 125 $\mu\text{g}/\text{ml}$) or with medium only. After stimulation, the cultures were centrifuged, the supernatants were collected, and the cells were subjected to extraction as described above. The supernatants and cell granule extracts were assayed for PR-39 by ELISA. PMA, LPS, and OZ released 20.80 \pm 1.81%, 4.51 \pm 0.25%, and 3.51 \pm 0.42% of the total amount of PR-39 (mean \pm SEM, $n = 3$), respectively.

To investigate the involvement of PR-39 during a bacterial infection, two *S. choleraesuis* experiments were conducted with a total of 18 pigs. Healthy, early-weaned pigs (10 to 14 days of age) were obtained from a commercial farm where neither clinical signs nor laboratory evidence of salmonellosis or any other enteric diseases had been observed. Pigs were housed in an environmentally controlled isolation facility in which the temperature was maintained at 25°C; pigs had access to feed and water ad libitum. After 7 days of acclimation to their new environment, the pigs were deprived of food for 6 h and then challenged orally with 9.5 $\times 10^9$ CFU of *S. choleraesuis* (kuzendorf). The *Salmonella* challenge isolate originated from the lung of a pig dying in a field outbreak of septicemic salmonellosis; the serotype identification was made by the National Veterinary Service Laboratory (Ames, Iowa). To prepare the challenge inoculum, *S. choleraesuis* was plated onto a blood agar plate and grown overnight at 37°C. A colony was inoculated in 5 ml of brain heart infusion broth, grown for 8 h, and then used to inoculate additional brain heart infusion broth, which was incubated for 12 h. The challenge inoculum (1.9 $\times 10^9$ CFU/ml) was given (5 ml) by gastric tube to each pig. Blood samples were collected from all pigs by venipuncture of the anterior vena cava with both EDTA-treated and non-treated vacuum tubes before challenge (day 0) and on various days thereafter. Serum samples were obtained by centrifugation within 1 h after collection, diluted (1:20) with 1% BSA in PBS containing 0.01% CETAB, and analyzed for PR-39 by the sandwich ELISA. The concentrations of serum haptoglobin, an acute-phase protein, were measured as previously described (2, 13). The total leukocyte numbers were determined with an electronic cell counter (Danam Electronic, Dallas, Tex.), and differential leukocyte counts were determined by counting 100 cells in Wright-stained blood smears. Fecal swabs and rectal

TABLE 1. Responses of pigs to *S. choleraesuis* infection^a

Day post-infection	Rectal temperature (°C)	Haptoglobin concn (µg/ml)	No. of pigs with fecal <i>Salmonella</i> (n = 8)
0	40.0 ± 0.1	169.9 ± 7.5	0
3	41.3 ± 0.2 ^b	820.0 ± 58.1 ^b	8
10	39.8 ± 0.1	680.4 ± 45.5 ^b	8
17	40.1 ± 0.1	438.4 ± 107.3	2

^a Pigs (17 to 21 days of age) were challenged orally with 9.5×10^9 CFU of *S. choleraesuis* (*kunzendorf*) on day 0. Values are means ± SEM (n = 8).

^b P < 0.01 compared with values for day 0 as calculated by the paired Student *t* test.

temperatures were obtained from each pig at each blood collection. The isolation of *Salmonella* from feces was conducted as described by Waltman et al. (24), with the following modifications: the enrichment was with Rappaport-Vassiliadis R10 broth (Difco Laboratories, Detroit, Mich.), and the bacteria were plated onto MacConkey and Hebtoen enteric agar plates (Difco).

On day 3 postinfection of experiment 1, the rectal temperatures were increased ($P < 0.01$) and the level of serum haptoglobin was elevated ($P < 0.01$) nearly fivefold compared to the day 0 values (Table 1), demonstrating a clear response to the *S. choleraesuis* challenge. On day 3 postinfection, a neutropenia was observed; however, regardless of the lower number of neutrophils, the concentrations of serum PR-39 were increased 56% ($P = 0.06$) compared to the day 0 values (Fig. 2A). The serum values of PR-39 at day 3 postinfection probably reflected the contribution of the intestinal inflammatory response to *S. choleraesuis* and the movement of the antibacterial peptide into the systemic circulation. Ten days after challenge, at the height of the bacterial infection as judged by neutrophil counts, the serum PR-39 concentrations were threefold greater ($P < 0.01$) than the prechallenge values (Fig. 2A). On day 17 after challenge, the rectal temperatures were approaching the basal values, and *S. choleraesuis* was detected in only two of eight pig fecal samples, indicating initial recovery from the infection; however, the serum PR-39 concentrations remained elevated ($P < 0.05$).

To investigate the reproducibility of these in vivo data and to further define the kinetic response of serum PR-39 to *S. choleraesuis* challenge, a second experiment was conducted. Experiment 2 was identical to experiment 1 except that 10 pigs were used, the challenge inoculum was 6.5×10^9 CFU/pig, and the samples were collected at weekly intervals for 4 weeks. In agreement with our earlier findings, the serum PR-39 concentrations increased threefold ($P < 0.05$) following infection with *S. choleraesuis* (Fig. 2B). The serum PR-39 concentrations began to decrease by day 21 and returned to basal values on day 28, when the pigs were clinically recovered from the infection. The serum PR-39 concentrations did not correlate well with blood neutrophil counts ($r = 0.51$ for experiment 1 and 0.56 for experiment 2), indicating that increased concentrations of circulating PR-39 might depend not simply on the number of neutrophils in the blood but also on neutrophil involvement at the sites of infection.

Stimulation of the neutrophils with PMA caused 20% of the total PR-39 to be released extracellularly. However, in vitro treatment with OZ or LPS induced relatively little release of PR-39 (3 to 5%), suggesting that PR-39 may kill bacteria primarily in phagosomes rather than extracellularly. This conclusion is supported by the in vivo findings in which the serum PR-39 concentrations on days 10 and 17 postchallenge reached

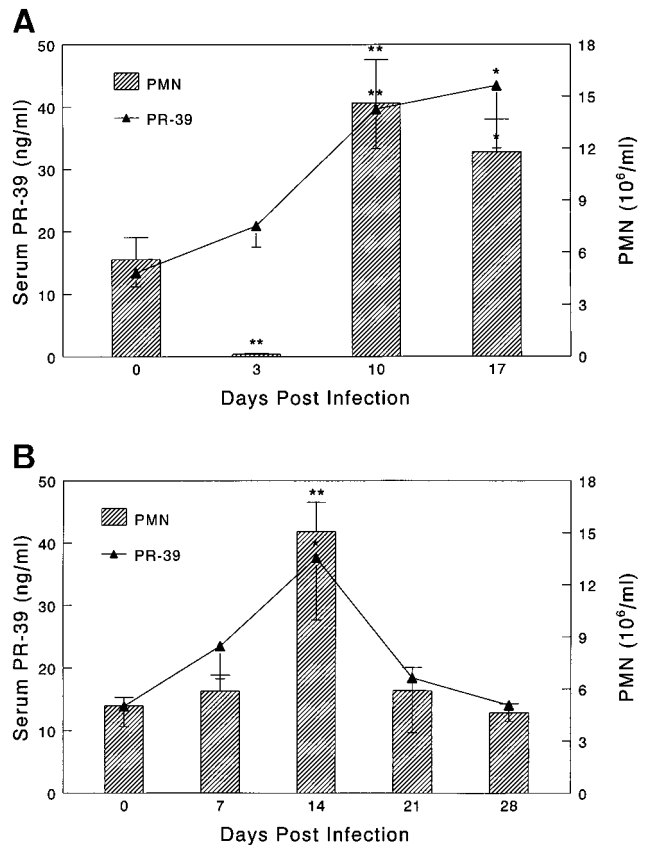


FIG. 2. Influence of *S. choleraesuis* infection in pigs on neutrophil numbers and concentrations of serum PR-39. Pigs (17 to 21 days of age) were housed in an environmentally controlled isolation facility and provided access to feed and water ad libitum. On the day of challenge (day 0), the pigs were deprived of food for 6 h and then challenged orally with *S. choleraesuis* (*kunzendorf*). Total leukocytes were counted on an electronic cell counter, and polymorphonuclear neutrophil (PMN) counts were determined by counting 100 leukocytes on Wright-stained blood smears. The concentrations of PR-39 in serum were measured by a sandwich ELISA. Values are means ± SEM. (A) Experiment 1, n = 8; (B) experiment 2, n = 10. * and **, $P < 0.05$ and $P < 0.01$ compared with value for day 0, respectively.

approximately 40 ng/ml, which is below the in vitro MIC of PR-39 for *S. choleraesuis* (2 µg/ml) (19). Earlier studies also showed that OZ induced <3% release of defensins from human neutrophils (5). Alternatively, because more than one antibacterial peptide likely is involved in host responses to a bacterial infection, systemic PR-39 at lower concentrations may act synergistically with other antibacterial peptides. Indeed, synergism among antibacterial peptides has been reported (19, 25); however, the contribution of circulating PR-39 to intravascular microbial killing is not yet clear. In summary, these findings suggest that the extracellular release of PR-39 can be regulated by exogenous stimulants and that the concentrations of serum PR-39 reflect the involvement of this antibacterial peptide in the microbicidal function of neutrophils during a *Salmonella* infection.

We thank Tomas Ganz and Jishu Shi of the Department of Medicine at the University of California, Los Angeles, for kindly providing the rabbit anticathelin antiserum; Shucheng Zhang and Hua Wu at Kansas State University for helpful advice in hybridoma production; and Danielle Goodband, Vicki Davidson, and Christy Davison for their excellent technical assistance.

This work was supported in part by USDA NRI Competitive Grant 95-37204-2141.

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