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Secretion of Type II Phospholipase A_2 and Cryptdin by Rat Small Intestinal Paneth Cells

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We examined the secretion of antimicrobial proteins and peptides into surgically isolated and continuously perfused segments of rat small intestine. Up to nine discrete antimicrobial molecules appeared in the intestinal perfusates following intravenous administration of bethanechol, a cholinergic agonist, or intralumenal instillation of lipopolysaccharide (LPS). Among them were three markers of Paneth cell secretion: lysozyme; type II (secretory) phospholipase A_2; and at least one intestinal defensin, RIP-3, that appeared to be an alternatively processed variant of the rat neutrophil defensin RatNP-3. Both bethanechol- and LPS-stimulated intestinal lumenal perfusates (washings) contained molecules that killed *Escherichia coli*, *Salmonella typhimurium*, and *Listeria monocytogenes* in vitro. These molecules were more active against the avirulent *S. typhimurium* strain 7953S (*phoP*) than against its virulent parent, *S. typhimurium* 14028S. These data demonstrate that small intestinal Paneth cells secrete antimicrobial peptides in vivo, that this secretion is regulated by the autonomic (parasympathetic) cholinergic nervous system, and that the release of antimicrobial molecules can be triggered by the presence of bacterial LPS in the intestinal lumen.

Although the principal role of the small intestine pertains to nutrition, certain portions serve other functions. For example, surface Peyer's patches conduct immunologic surveillance and the vital processes of epithelial cell renewal take place in tubular structures (crypts) that are recessed below the lamina propria. Because maintaining the integrity of villous epithelium is important for nutrition and for host defense and because replicating cells are especially vulnerable to damage by prokaryotic organisms and viruses, the defenses of intestinal crypts against potential pathogens are of considerable interest.

It has been suggested that responsibility for this function falls upon Paneth cells, which are located at the base of these crypts and contain an array of antimicrobial molecules that includes cryptdins (intestinal defensins) (3, 11, 17, 24, 25, 34), lysozyme (1, 26, 29), and type II phospholipase A_2 (PLA_2) (9, 13, 21, 22, 35). Recently, our laboratory described the structures and in vitro antimicrobial activities of several antimicrobial polypeptides derived from murine Paneth cells, including cryptdins (3) and type II PLA_2 (9). We have now developed a system to study the secretion of antimicrobial molecules from rat Paneth cells in vivo. This report describes the model and our discovery of a new rat intestinal defensin.

MATERIALS AND METHODS

Rat intestinal perfusate collection. Forty-seven male Sprague-Dawley rats, 180 to 210 g, were subjected to fasting overnight from food but not water. After they were anesthetized intraperitoneally with pentobarbital, 100 mg/kg of body weight, appropriate ventilation was established. The abdomen was opened, and the proximal intestine and distal ileum were identified and incised. A polyethylene PE50 catheter was introduced into the proximal small intestine, about 15 cm distal to the duodenal-colic ligament of Treitz, and secured. The intestine was gently flushed with 10 to 20 ml of 0.9% saline. A larger-bore (PE280) tubing was placed into the distal ileal incision and tied into place. This isolated the small intestinal segment from salivary, gastric, pancreatic, or biliary secretions and allowed intestinal perfusates to be collected directly into an ice-cold receptacle. During the injections (described below) and for the following 25 min, the intestine was perfused through the PE50 catheter with 30 ml of 0.9% saline with the aid of a Harvard Instruments infusion pump.

The rats were divided into three groups. Group A rats ($n = 34$) received a slow intravenous infusion of bethanechol, 0.5 mg/kg of body weight, over approximately 5 min before beginning intestinal lumenal perfusion. Group B rats ($n = 6$) were exposed to intralumenal lipopolysaccharide (LPS) by incorporating 10 μg of LPS per ml (*Escherichia coli* O127:B8; Difco Laboratories, Detroit, Mich.) into the saline perfusate solution. Group C rats ($n = 7$) received a slow intravenous injection of saline and served as the controls.

Dialysis and protein concentration. Rat intestinal perfusates recovered from the above procedures were individually placed in Spectra/Por 3 dialysis bags (Spectrum, Houston, Tex.) with a nominal molecular mass cutoff of 3,500 Da and were dialyzed against 1% acetic acid at 4°C for 18 h. Postdialysis materials were concentrated to a volume of 1 to 2 ml by vacuum centrifugation in a Speed Vac Concentrator (Savant Instruments, Hicksville, N.Y.). The concentrated supernatants were removed, their protein contents were determined by bicinchoninic acid assay (36), and they were stored frozen until used.

PLA_2 activity. Autoclaved *E. coli* ML 35 labeled with [^{14}C]oleic acid (NEN Dupont; 50 mCi/mmol) was used as PLA_2 substrate (5). Each rat intestinal perfusate (5 μg of protein) was mixed with 50,000 cpm of ^{14}C-labeled *E. coli* (equivalent to 2.5×10^8 bacteria, adjusted by adding nonradioactive *E. coli*) in a 250-μl volume that contained 250 mM Tris, 10 mM $CaCl_2$, and 1 mg of bovine serum albumin per ml, pH 9.5. After incubation in a 37°C shaking water bath for 15 minutes, 100 μl of 2 N HCl was added to stop the reaction, and the product (free fatty acids and lysophospholipids) was trapped by adding 100 μl of 20-mg/ml fatty acid-free bovine serum albumin. The mixture was kept at 4°C for 30 min and centrifuged at $10,000 \times g$ for 5 min, and the pellet was washed twice. PLA_2 activity was measured by liquid scintillation counting of [^{14}C]oleic acid in the combined supernatants and converted to arbitrary units (1 U = 1% release of ^{14}C).

Lysozyme activity. Lysoplates (23) contained 1% agarose and 0.5 mg of lyophilized *Micrococcus lysodeikticus* per ml in 15 ml of 66 mM sodium phosphate buffer, pH 7.0. Samples of each concentrated intestinal perfusate, containing 5 μg of protein dissolved in 5 μl of 0.01% acetic acid, were placed into 3-mm-diameter wells and incubated overnight at room temperature. Lysozyme activity of the rat intestinal perfusate was expressed relative to hen egg white lysozyme, which was used as the standard.

Antimicrobial activity-gel overlay assay. Gel overlay assays (16) were performed by running the concentrated samples on acid-urea (AU)–12.5% polyacrylamide gel electrophoresis (PAGE) gels that were rinsed with 10 mM sodium phosphate buffer (pH 7.4) and then placed on top of thin agarose gels containing viable bacteria. After 3 h, the AU-PAGE gel was removed and replaced by an agar overlay (60 g of Trypticase soy agar per liter). The 3-h transfer period before pouring the overlay gel allowed antimicrobial peptides to diffuse from the AU-PAGE gel into the underlay agar. After overnight incubation at 37°C to allow bacterial colony formation, the location of antimicrobial peptides in the perfusates was made evident by corresponding clear zones on the plate. Test organ-

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TABLE 1. Composition of rat intestinal perfusates[a]

Treatment	Group	Animals (*n*)	Total protein (mg)	PLA_2 activity (U)		Lysozyme activity (U)	
				Total	Specific	Total	Specific
Bethanechol	A	34	1.05 ± 0.08***	2,024.1 ± 201.5***	1,949.2 ± 144.7***	24.1 ± 8.41 (NS)	16.1 ± 4.94 (NS)
LPS	B	6	2.99 ± 0.68**	2,136.2 ± 234.6***	912.6 ± 219.4*	28.3 ± 7.20**	7.90 ± 1.61***
Saline control	C	7	0.26 ± 0.05	76.7 ± 29.4	362.4 ± 121.0	0.15 ± 0.10	0.49 ± 0.32

[a] All data are expressed as means ± standard errors of the mean. One unit of PLA_2 activity equals 1% release of ^{14}C, determined by measuring the release [^{14}C] oleate from ^{14}C-labled *E. coli*. One unit of lysozyme activity is equivalent to the activity of 1 μg of hen egg white lysozyme, as determined in a radial diffusion (lysoplate) assay. Specific activity values are expressed as units per milligram of protein. Statistical significance was determined by Student's *t* test. Bethanechol- and LPS-treated rats were compared with saline controls. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; NS, not significant.

isms included *Listeria monocytogenes* EGD, *E. coli* ML 35p, *Salmonella typhimurium* 14028S (mouse virulent), and its avirulent *phoP* derivative *S. typhimurium* 7953S.

Other methods. Protein composition was analyzed with sodium dodecyl sulfate (SDS)–16.5% PAGE and AU–12.5% PAGE minigels. The antimicrobial peptide provisionally named RIP-3 was purified by continuous AU-PAGE (8) and reversed-phase high-pressure liquid chromatography (HPLC), and its sequence was determined with a Porton Model 2090E sequencer (Beckman Instruments,

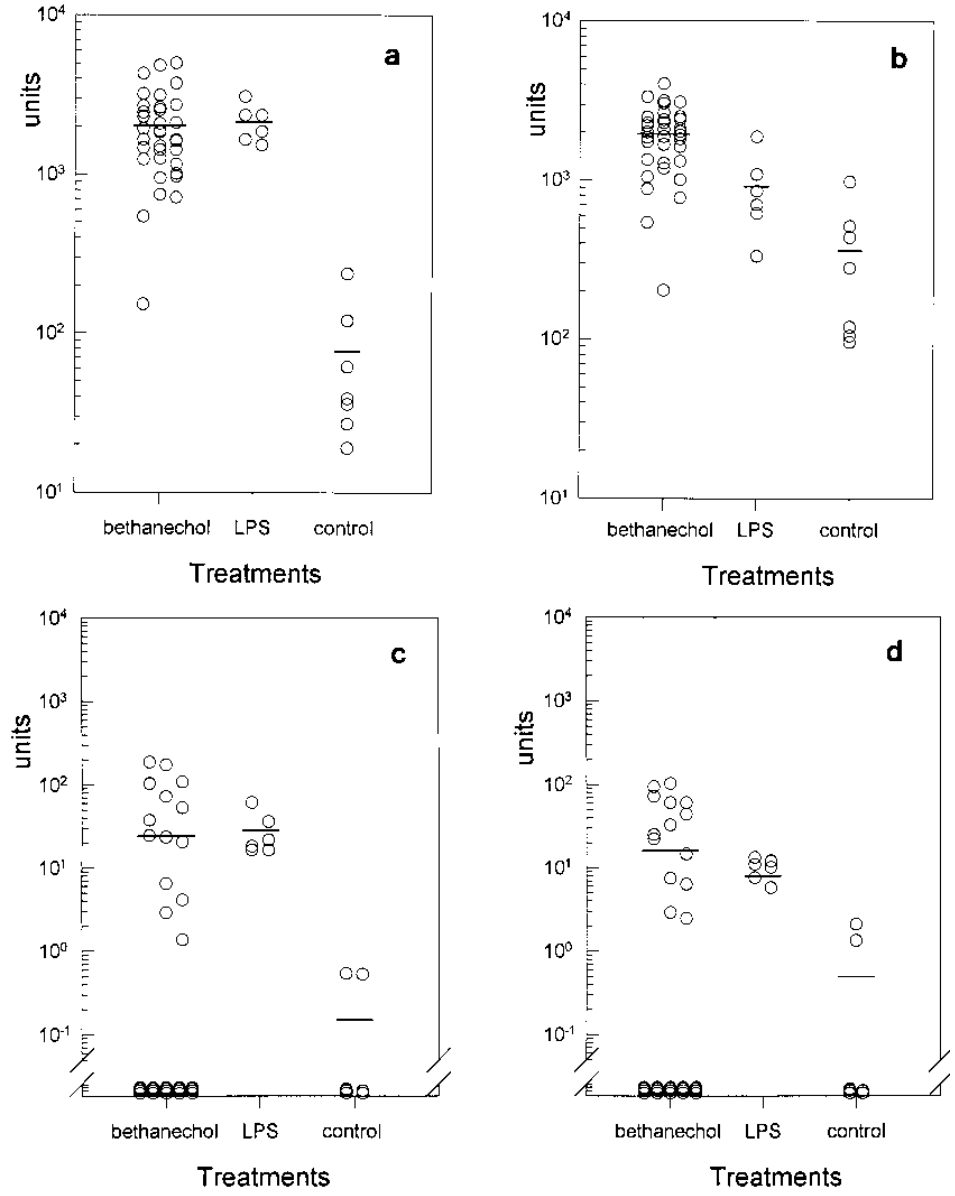

FIG. 1. PLA_2 and lysozyme activity of rat intestinal lumenal perfusates. (a and b) Total and specific activity, respectively, of PLA_2. (c and d) Total and specific activity, respectively, of lysozyme. Horizontal lines indicate the mean values for each group: bethanechol-stimulated rats ($n = 34$), LPS-stimulated rats ($n = 6$), and saline-treated controls ($n = 7$).

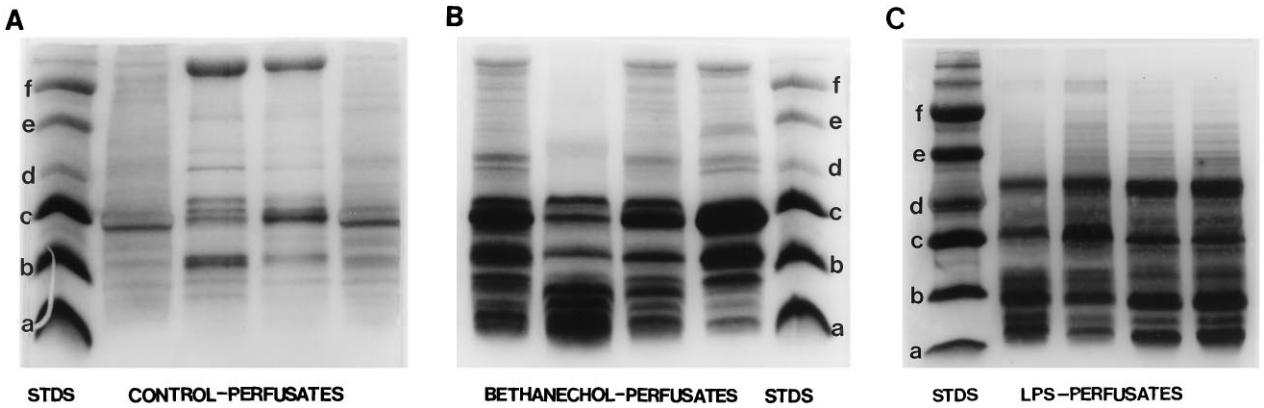

FIG. 2. Silver-stained SDS–16.5% PAGE gels. Each group contains perfusates from four animals, with 20 μg of protein applied per lane. The left lane (A and C) or right lane (B) shows molecular mass standards: 3.0 (a), 6.2 (b), 14.3 (c), 18.4 (d), 29.0 (e), and 43.0 (f) kDa.

Fullerton, Calif.) after it had been reduced, carboxymethylated (3), and transferred to a polyvinylidene difluoride membrane (18). Mass spectrometric measurements (10) were done on a PerSeptive Biosystems matrix-assisted laser desorption ionization (MALDI)–mass spectrometry instrument.

RESULTS

The contents of the intestinal perfusates collected from all of the animals are described in Table 1. Intestinal perfusates from bethanechol-treated rats contained four times more protein ($P < 0.001$) and over 25-fold more PLA_2 ($P < 0.001$) than the control perfusates. Intestinal perfusates obtained from LPS-treated animals contained 11.5-fold more protein ($P < 0.01$) and 27.8-fold more PLA_2 activity ($P < 0.001$) than control perfusates.

The activities of PLA_2 and lysozyme in the individual intestinal perfusates are shown in Fig. 1. PLA_2 activity was detected in every perfusate, and its level increased uniformly after stimulation by bethanechol and LPS. It has recently been demonstrated that type II PLA_2 can kill gram-negative and gram-positive bacteria, including *L. monocytogenes*, *E. coli*, and *S. typhimurium* (9, 37).

In contrast to the uniform PLA_2 response, lysozyme activity in the perfusates was more variable. Only two of the seven perfusates from saline-treated controls had detectable levels, and even these were quite low (0.54 ± 0.01 total units, mean ± standard error of the mean; $n = 2$). Much higher levels of total lysozyme activity (28.3 ± 7.2 U, $n = 6$) were present in perfusates from the LPS-treated rats ($P < 0.001$). Many of the bethanechol-induced intestinal perfusates contained considerable amounts of mucus, a densely polyanionic polymer (28). Probably as a consequence of this (lysozyme is strongly cationic and its binding to mucus would prevent its diffusion in lysoplate assays), only 14 of the 34 perfusates from bethanechol-treated rats had measurable lysozyme levels. In these samples, levels of lysozyme activity (62.6 ± 16.7, mean ± standard error of the mean; $n = 14$) exceeded those found in the LPS-induced perfusates.

It was clear from inspecting the 16.5% Tricine SDS-PAGE gels shown in Fig. 2 that the composition of intestinal perfusates from control and experimental rats differed in important respects. Although some heterogeneity of content was noted, control perfusates (Fig. 2A) typically contained meager amounts of peptides smaller than lysozyme (14.3 kDa). In contrast, specimens obtained after stimulation with bethanechol (Fig. 2B) or LPS (Fig. 2C) contained a plethora of such peptides, including several in the 3- to 6-kDa size range.

We analyzed these perfusates for molecules with antimicrobial activity by performing gel overlay assays. In such assays, the perfusates were first electrophoresed on AU-PAGE minigels and then rinsed and blotted on top of underlay gels uniformly impregnated with viable bacteria. In the overlay assays shown in Fig. 3, 1 μg of rabbit neutrophil defensin NP-1 was used as a positive control, and it appears in the left lane of each panel. After removing the minigels, an overlay agar was poured and the bacterial gels were incubated to allow the surviving organisms to form colonies.

The clear zone caused by blotting NP-1 corresponded precisely with its location, just behind the dye front, in corresponding gels that were stained with Coomassie blue. The middle lanes in each panel contained 40 μg of protein that was derived from control perfusates, and the right lanes contained 40 μg of protein from LPS-induced perfusates. It can be seen that the LPS-induced perfusates contained multiple components that killed *E. coli* ML 35p (Fig. 3b) and *S. typhimurium* 7953S (Fig. 3c). In contrast, control perfusates lacked such activity. Fewer components of the LPS-stimulated perfusate showed activity against *L. monocytogenes*, a gram-positive bacterium (Fig. 3a). Note that *S. typhimurium* 14028S (Fig. 3d), the mouse-virulent progenitor of *S. typhimurium* 7953S, was considerably more resistant to many of these peptides than was its avirulent *phoP* transformant, 7953S.

To estimate how many discrete antimicrobial molecules

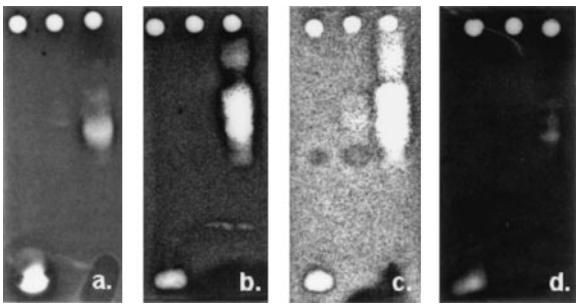

FIG. 3. Gel overlay assays with perfusates from LPS-treated rats. (a) *L. monocytogenes* EGD; (b) *E. coli* ML 35p; (c) *S. typhimurium* 7953S; (d) *S. typhimurium* 14028S. In each panel, the left lane contained 1 μg of rabbit defensin NP-1 (a positive control), the middle lane contained 40 μg of control intestinal perfusate protein (pooled from six rats), and the right lane contained 40 μg of LPS-stimulated intestinal perfusate protein (pooled from three rats). The gels were stained with Coomassie blue and dried before photography.

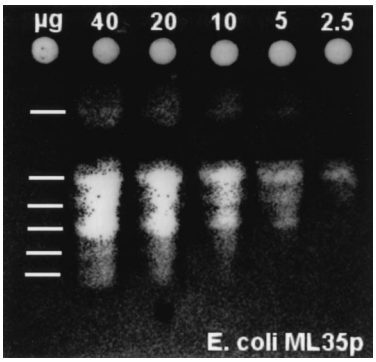

FIG. 4. Gel overlay assay with perfusates from LPS-treated rats. The test organism was *E. coli* ML 35p. Serial twofold dilutions of pooled perfusate containing, from left to right, 40, 20, 10, 5, and 2.5 μg of total protein per lane are shown. At least six distinct clear zones (indicated by the white lines), corresponding to different molecules, can be seen.

were present in the LPS-stimulated intestine perfusate, we exposed the sensitive indicator strain, *E. coli* ML 35p, to serial twofold dilutions of perfusate protein in a gel overlay assay (Fig. 4). At least six distinct bands with microbicidal activity were apparent. Identical results were obtained when we used *S. typhimurium* 7953S as the target (data not shown).

The bethanechol-stimulated perfusates were subjected to a similar analysis, after identifying samples with especially well-defined banding patterns for the studies. The bethanechol perfusates contained three highly cationic antimicrobial components in addition to those seen in the LPS-stimulated samples. Each of these more cationic components was effective against *L. monocytogenes*, *E. coli*, and *S. typhimurium* 7953S, but none had substantial activity against *S. typhimurium* 14028S (Fig. 5). By SDS-PAGE, each of these components had an apparent mass of approximately 4 to 5 kDa (data not shown).

To determine if any of these cationic molecules were cryptdins (intestinal defensins), most of the bethanechol-treated perfusates were pooled and subjected to continuous preparative AU-PAGE. Fractions enriched in cationic 4- to 5-kDa components were identified by performing AU and SDS-PAGE gels, and these fractions were further purified by reversed-phase HPLC. Rat intestinal peptide-3 (RIP-3), the peptide described below, had a mass of 3,594.4 Da by MALDI mass spectrometry. Microsequencing provided unambiguous assignments for 27 of its first 29 N-terminal residues. Except for its four-residue N-terminal extension (Arg-Gly-Val-Thr),

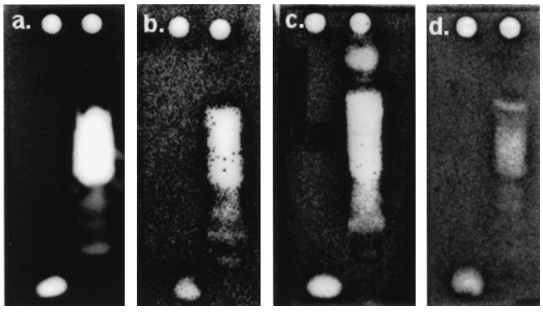

FIG. 5. Gel overlay assays with perfusates from bethanechol-treated rats. (a) *L. monocytogenes* EGD; (b) *E. coli* ML 35p; (c) *S. typhimurium* 7953S; (d) *S. typhimurium* 14028S. In each panel, the left lane contained 1 μg of rabbit defensin NP-1 (a positive control) and the right lane contained 40 μg of bethanechol-stimulated intestinal perfusate protein (pooled from three rats). The gels were stained with Coomassie blue and dried before photography.

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"RIP-3"   AGVTXSCRTS SCRFGERLSG ACRLNGXIYX XXX  (Mr = 3594.4) *
          |||| ||||| :|||||||:|| ||||||  ||
RatNP3    agvtCSCRTS SCRFGERLSG ACRLNGRIYR LCC  (Mr - 3594.2)**
          ||||  '|    | | ||||| ||   ;  ||
RatNP1    agvtCYCRRT RCGFRERLSG ACGYRGRIYR LCCR
          ||||  ||    | | :|||| ||   ;  ||
RatNP2    agvtCYCRST RCGFRERLSG ACGYRGRIYR LCCR
          ||    ||    | | |||||  |:   |  ||
RatNP4    agqaCYCRIG ACVSGERLTG ACGLNGRIYR LCCR
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FIG. 6. Primary sequences of RIP-3 and four rat neutrophil defensins (Rat NPs). Amino acids are represented in standard single-letter code. Lowercase letters denote residues that are present in the precursor prodefensin but absent in the mature (processed) peptide. X denotes an unidentified residue. *, MALDI-mass spectrometry mass of purified RIP-3. **, Calculated mass of the nonoxidized form of RatNP-3 plus the amino-terminal AGVT residues. RatNPs 1 to 4 and proRATNP-3 are described in references 2, 4, and 39.

the primary sequence of RIP-3 was identical to that of a previously described rat neutrophil defensin, RatNP-3 (4) (Fig. 6). Since the N-terminal extension AGVT is present in pro-RatNP-3 (39) and the masses are in satisfactory agreement, we believe that RIP-3 is the Paneth cell's version of mature (i.e., processed) proRatNP-3. The antimicrobial properties of defensins have been well characterized (15).

DISCUSSION

Although Paneth cells were first described over a century ago and have been observed in many mammals including humans, rabbits, rats, and mice (38), this is the first study to test their in vivo secretion of antimicrobial polypeptides directly. Type II PLA_2, intestinal defensins, and lysozyme all exist in the Paneth cell's granules and therefore afford useful markers of Paneth cell secretion (1, 3, 9, 11, 13, 22, 24, 26, 29, 35).

We found that stimulated rat Paneth cells secreted a plethora of small antimicrobial peptides, including the above markers and many other 3- to 14-kDa molecules which remain to be identified. In conjunction with earlier ultrastructural evidence obtained from murine models (30–32), these findings indicate that Paneth cell secretions can play a central role in protecting the small intestinal crypts.

The finding that virulent *S. typhimurium* 14028S was less susceptible to many of these secreted intestinal microbicides than its isogenic *phoP* derivative, 7953S (6), is consistent with a considerable body of evidence suggesting that the pathogenicity of *Salmonella* spp. is governed, at least in part, by *phoP*-associated resistance to defensins and other antimicrobial proteins (7, 19, 20).

That vagal activity is stimulated by the ingestion of a meal is well established. Since the passage of food mixed with oral secretions can introduce microorganisms into the small intestine, cholinergic regulation of Paneth cell secretion provides a regulatory system that can mobilize antimicrobial peptides into the intestinal crypts when these structures are most likely to be challenged by the entry of microbes.

We found that instilling LPS into the intestinal lumen induced a noteworthy release of at least six antimicrobial molecules that could kill *E. coli* ML 35p and *S. typhimurium* 7953S. Several additional antimicrobial molecules were found in perfusates collected after bethanechol administration. LPSs are prominent components of the outer membranes of gram-negative bacteria that can elicit the production of mediators, such as interleukin-1 and tumor necrosis factor (14, 27). Consequently, it would be interesting to know whether the signals that inform Paneth cells of the presence of LPS in the small intestinal lumen are transmitted directly to Paneth cell receptors for LPS, arrive via the autonomic nervous system, or are

transmitted by cytokines or hormone-like messengers. It is curious that tumor necrosis factor mRNA exists in murine Paneth cells (12) and that mature tumor necrosis factor has been immunolocalized to the peripheral regions of their secretory granules (33). Although this observation is of uncertain significance for the Paneth cell's secretory response, the presence of endogenous tumor necrosis factor alpha provides another functional nexus between Paneth cells and bacterial LPS.

Whatever the signals that inform Paneth cells of the presence of bacteria, our data suggest that microorganisms venturing into intestinal crypts are likely be greeted by a biochemical barrage of antimicrobial peptides and proteins.

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