Germ-free and Colonized Mice Generate the Same Products from Enteric Prodefensins*

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The use of germ-free mice offers the possibility to study antibacterial components in a gut uncolonized by bacteria. We have developed a method to extract and high pressure liquid chromatography-fractionate the antibacterial factors present in the small intestine of a single mouse. By mass spectrometry and sequence analyses of fractions exhibiting antimicrobial activity, we identified and characterized the defensin region in germ-free mice as well as in colonized mice. Defensins made up around 15% of the total antibacterial activity both in germ-free and colonized mice. The intestine of germ-free mice exhibited the same set of mature enteric defensins (defensins 1, 2, 3, 4, and 6) as mice colonized by a normal microflora. Mature defensins are generated through processing of larger precursors by enzymatic removal of a signal peptide and a propiece. We found that all prodefensins were cleaved at a Ser/Ala-Leu bond, giving 34-residue propiece peptides and only trace amounts of the predicted 39-residue peptide. This first step must be followed by the removal of a residual peptide to render the mature defensins, indicating that the processing is more complex than previously anticipated. The same propieces were found in both germ-free and colonized mice, suggesting that the same processing operates independent of bacterial presence in the intestine.

...subject to further processing, rendering an anionic propiece of unknown function and the cationic mature defensin (5). Unlike humans, mice lack granular defensins in their neutrophils (6). However, cloning and sequencing predicted the mouse small intestine to produce at least 17 different defensins (cryptdins) (4, 7), but thus far, only 6 of these putative peptides have been isolated and studied in vitro (8–10).

All mammals have a normal microflora in the gastrointestinal tract that is most numerous in the colon. The small intestine has a much lower number of bacteria, most likely due to Paneth cell production of enteric defensins, lysozyme and phospholipase A₂ (8, 11–13). Here we developed a procedure for extraction and high pressure liquid chromatography separation of the antibacterial factors present in the small intestine of a single mouse. We show that the enteric defensins represent but one set of antimicrobial components present in mice small intestine. By comparing germ-free mice to mice with a normal microflora, we demonstrate that microbes are not necessary for the processing and production of enteric defensins. Furthermore, the differences in active defensins in the small intestine between colonized and germ-free mice are smaller than the differences between individual animals.

EXPERIMENTAL PROCEDURES

Animals, Their Maintenance, and Infections—Germ-free mice of the NMRI/KI strain have been inbred for 43 generations under conditions described previously (14) and approved by the Swedish Board of Agriculture (Permission Numbers 4233060 and 35-4114/99). To avoid genetic drift and to allow comparisons with mice having a normal flora, the microflora is reintroduced at regular intervals to some germ-free mice by feeding them feces from colonized mice. These mice are bred in parallel for several generations. Such animals are referred to as conventional mice. Microbial status of germ-free mice was controlled initially and at the termination of the experiments. All NMRI mice received the same autoclaved rat diet (R36; Lactamin, Vadstena, Sweden) and sterile water. C3H/HeJ mice were purchased from Jackson Laboratories and kept in our breeding unit for 4 weeks before the onset of experiments.

Peptide Extraction, HPLC Purification, and Analysis of Purified Components—The small intestines, which were assumed to contain 80% water, were ground in liquid nitrogen, and the frozen powder thus obtained was extracted for 10 min (repeated vortexing for 2 min) with ice-cold 60% aqueous acetonitrile containing 1% trifluoroacetic acid. The extracts were centrifuged at 11,000 × g for 20 min. Supernatants were freeze-dried, redissolved in water with 20% ethanol, and cleared by centrifugation. The extracts were characterized by reverse phase-HPLC. The amounts of material applied corresponded to 0.15 g of tissue. Elution was performed with 0.1% trifluoroacetic acid in H₂O (solvent A) and a gradient of 0.15% trifluoroacetic acid in acetonitrile (solvent B) on a 0.46 × 25-cm Vydac C18 column (The Separation Group, Hesperia, CA) with a flow rate of 0.8 mL/min. Initial conditions...
were 5% solvent B for 15 min, 5–52% solvent B for 94 min, and, finally, 52–95% solvent B for 5 min. Fractions of 0.8 ml were collected and lyophilized. Freeze-dried chromatographic fractions were redissolved in 10 μl of 20% ethanol. N-terminal sequence analyses were performed by Edman degradation in Proceice cLC or HT instruments (PE Applied Biosystems). Matrix-assisted laser desorption/ionization (MALDI) time of flight mass spectrometry analysis was carried out using a Voyager-DE PRO Biospectrometry Workstation (PerSeptive Biosystem Inc.). One μl of each fraction was mixed with 1 μl of matrix solution (α-cyano-4 hydroxycinnamic acid) in 60% acetonitrile/0.1% trifluoroacetate acid. The spectra were acquired in the linear or reflectron mode. Normally, mass values were detected as the average masses using the linear mode, which, in general, are a few daltons higher than the monoisotopic mass values. The complexity of the sample (several defensins and propieces) and low peptide ionization, especially for defensins, did not permit us to routinely use the reflectron mode to determine monoisotopic masses. Calibration was performed using external calibration.

**Bacterial Strains and Assays for Antibacterial Activity—Antibacterial activity was recorded with an inhibition zone assay (15).** Briefly, thin plates were poured containing Luria-Bertani broth, 1% agarose, and about 3 × 10^7 log phase bacteria (Bacillus megaterium, strain Bm11 from our own collection). Small wells were punched in the assay plates (3 mm in diameter) and loaded with 2.6 μl of sample. After an overnight incubation at 30 °C, the diameter of inhibition zones was recorded. Cecropin units were read from a standard curve obtained with cecropin A, and 1 unit corresponds to the activity of 1 ng of cecropin A.

**RESULTS**

**Defensins Represent a Minor Part of the Total Antimicrobial Activity from the Small Intestine in GF as well as in Conventional Mice—**Most data on the functions of antimicrobial peptides are derived from in vitro experiments. To approach the in vivo situation, we developed a procedure allowing us to analyze the antimicrobial components in the small intestine of a single mouse (about 1 g of tissue). This was accomplished by an extraction with only volatile solvents followed by freeze-drying, redissolving, and HPLC separation. Fig. 1 shows chromatographic analyses of the antibacterial factors present in the small intestine of a conventional mouse and a germ-free mouse (each corresponding to about 0.15 g of tissue). The active fractions were investigated with MALDI mass analysis and by Edman microsequencing.

The defensins accounted only for about 15% of the total antimicrobial activity (Fig. 1). Several of the other antimicrobial factors have been tentatively identified and will be reported elsewhere. Here we only discuss the defensin region of the HPLC chromatograms. We have analyzed more than 27 individual mouse intestines, and the overall reproducibility of the chromatograms as shown in Fig. 1 is good. However, individual variations were detected when the defensin part of the chromatogram was enlarged. Fig. 2 shows the defensin regions in chromatograms from three conventional mice and three GF mice. The chromatographic profile of all six mice shows a major peak at 57–58 min (Fig. 2). The N-terminal sequence AEIXFDXSK and a mass value of 4964 Da identified this component as thymosin β-4 (16). Because this peak is very well defined in each mouse, we used it as an internal standard.

**Identification of Defensins in HPLC Fractions from the Small Intestine of Both GF and Conventional Mice—**N-terminal sequencing (15 cycles) of the material in fractions 55–56 and 58–59 showed sequences (LRDLYVXYXRT/S/K/ARGXKXG) identical to mature enteric defensins. The X represents undetectable cysteine because in an unreduced sample, it gives rise to a pPM in the sequence. The amount analyzed was around 10–40 pmol, which enabled the identification of defensins (DEF 1, DEF 2, and DEF 3) based on a T, S, or K residue detected in position 10 (Fig. 3A). The major enteric defensin in fractions 58–59 was identified as DEF 6, based on an Ala residue in position 10, and the major MALDI peak of this fraction, m/z 4133, is in agreement with the expected value of 4131 Da for DEF 6. The most intense MALDI peaks and the expected masses of mature defensins are given in the right part of Table I. DEF 4 was detected as a minor mass peak in fraction 55–56, whereas DEF 5 with a predicted mass value of 4315 Da could not be detected. However, we did notice a component with a mass of 4229 Da that could represent DEF 5 with the first amino acid deleted. Taken together, we detected DEF 1–4 and DEF 6 in both germ-free as well as colonized mice (Table I, right part). The differences in the overall profile of defensins are smaller than the variations between individual animals (Fig. 2). It is known that synergy may be expected among the peptides, and the specific repertoire of one individual compared with the other may influence our total activity results.

The only qualitative difference found in the defensin region between GF and conventional mice was a novel peptide detected solely in fractions 60–61 of conventional mice. The N-terminal sequence LQDAAVGMAXPXR is identical to the translated cDNA sequence for CRS4C-4, a 38-residue peptide with 9 cysteines (17). The sample had detectable antibacterial activity, but because it was not judged as 100% pure, the activity could come from other components. In the correspond-
ing fractions from germ-free mice, no significant antibacterial activity was recorded.

**Processing of Defensin Propeptides**—Defensins are produced as inactive precursors that are cleaved proteolytically, yielding the active mature defensins and propiece peptides. N-terminal sequencing of fraction 53–56 gave almost identical sequences, DPIQNTDEETKTQ (the first time) and DS/PQNTDEETKTEXQ (the second time) (15 cycles). Both sequences are fully consistent with the peptides of the precursors given in Fig. 3A, except for DEF 5. The MALDI analysis is compatible with masses corresponding to propiece peptides of 34 residues. The known sequences for the enteric prodefensins show that amino acid exchange occurs at only 7 of 34 residues; thus, several propiece peptides will have identical or quite similar mass values. The MALDI data obtained and the expected masses for the propiece peptides are given in the left part of Table I. The majority of the UV-absorbing material in the defensin region eluting at 52–54 min is the 34-residue products, whereas traces of 39-residue propeptides (DEF 1, DEF 2, DEF 3, and DEF 6) were observed in fractions 55–60 (Figs. 2 and 3C). Mass values of fractions 60–62 corresponding to N-terminally elongated DEF 1 (mass, 4717 Da; theoretical mass, 4720 Da) and DEF 6 (mass, 4737 Da; theoretical mass, 4734 Da) were detected, but the amounts and the complexity of the samples did not allow confirmation by sequencing.

**Identification of Enteric Prodefensins in GF and Conventional Mice**—Several components elute after the defensin region. Fractions 65–69 contained no detectable antibacterial activity but had masses corresponding to those of prodefensins. As seen in Table II, the molecular masses agree well with the presence of prodefensins 1–6 lacking the signal sequence. This includes DEF 5 and DEF 4, which were difficult to detect as mature peptides, suggesting that the processing of mature DEF 5 in our mice is low or different from that reported previously. Reverse transcription-polymerase chain reaction of the germ-free mice also confirmed the presence of DEF 4 and DEF 5 mRNA.² Again, there were no significant differences in the prodefensin region of the HPLC chromatogram between GF and conventional mice.

**DISCUSSION**

To increase the understanding of the biological functions of antimicrobial peptides (peptide antibiotics), *in vivo* experiments are needed. Analysis of animals reared for many generations under sterile conditions (the germ-free mice) may provide the default levels and settings of antimicrobial components in the absence of microbes (18). In earlier biochemical studies of enteric mice defensins, pooled material and several enrichment steps were been used before the final separation. Compared with these studies, our approach allows an estimation of the overall number of components present in the small intestine of a single mouse and the variation between

² M. Hornef, unpublished observations.
individuals. The mice were not subjected to any stimulus, and thus the peptides produced represent a steady-state level. It was unexpected that the enteric defensins should account for only 15% of the total activity. However, it must be stressed that we have used one antibacterial assay that is not necessarily optimal for all antimicrobial substances.

The main conclusion of our work is that the presence or absence of an intestinal microflora does not have a major influence on the production of defensins. In the small intestine of GF mice, we detected enteric defensins DEF 1, DEF 2, DEF 3, and DEF 6 and low levels of DEF 4. The colonized conventional mice also exhibited the same set of defensins. This is in agreement with earlier findings with normal mice (8, 10). We did not detect mature DEF 5 in any of the mice analyzed, although the purification of this peptide has been reported previously (10). Low levels of defensin 5 mRNA were found in the mice we studied, and we could detect prodefensin 5 (Table II). The production of DEF 2 and DEF 3 was found to be linked to the induction of chloride secretion and crypt flushing, activities that DEF 4 and DEF 5 lack (19). Thus, proDEF 5 may be differentially regulated by a proteolytic processing, due to tentative function(s) other than microbicidal.

The conventional mice differed from GF mice in the production of CRS4C-4. To our knowledge, this is the first time mature CRS4C-4 peptide has been isolated. The peptide has an unusual composition with 9 cysteine residues, and its biological function is not yet known. No CRS4C mRNA was detected in newborn mice; however, newborn mice did have high levels of defensin 6 mRNA (17). These findings are similar to our results with GF mice, showing high levels of the propiece for DEF 6, but no detectable CRS4C-4.

The mouse enteric defensins are processed from 73-residue precursors. Our data show that there are at least two processing sites involved. We suggest that a major pathway includes a primary cut giving a 34-residue propiece, followed by a second cut trimming away the 4–7 additional residues to render the mature defensin (Fig. 3A).

**Fig. 3.** Primary sequences of prodefensins and identified processing products to render mature defensins. A, primary sequence of prodefensin 1–6 (DEF 1–6) (10). Gaps represent cleavage sites. The extra gap in the mature DEF 4 peptide indicates an additional cleavage site (8). B, the deduced sequence of CRS4C-4 (28). The gap represents the propiece cleavage site. C, processing pattern of prodefensins. Bold lines indicate the identified peptides, and the dotted line represents a potential peptide that has not been detected. Dashed lines mark the possible intermediates found in trace amounts.

**TABLE I**

MALDI identification of enteric defensins and their corresponding propieces, all of which are found in both germ-free and conventional mice

| Propiece of 34 amino acids | Mature defensin |
|---------------------------|-----------------|
| Expected Da | Observed MALDI Da | Expected Da | Observed MALDI Da |
| ppDEF 1 | 3653 | 3653 | DEF 1 | 4117 | 4119–4120 |
| ppDEF 2 | 3652 | 3653 | DEF 2 | 4248 | 4249 |
| ppDEF 3 | 3638 | 3639 | DEF 3 | 4275 | 4276–4278 |
| ppDEF 4 | 3623 | 3626 | DEF 4 | 3757 | 3758 |
| ppDEF 5 | 3662 | (3662) | DEF 5 | 4315 | 4315 |
| ppDEF 6 | 3667 | 3668 | DEF 6 | 4131 | 4131–4133 |
TABLE II
Identification of enteric prodefensins

| Prodefensin | Expected Da | Observed MALDI Da |
|-------------|-------------|-------------------|
| 1           | 8343        | 8359–8364         |
| 2           | 8474        | 8481–8486         |
| 3           | 8488        | 8481–8486         |
| 4           | 8231        | 8240              |
| 5           | 8445        | 8447              |
| 6           | 8371        | 8372              |

The propiece is the absolutely dominating compound. Only trace amounts of the 39-residue peptide were found. ii) This processing is similar to what has been found for other antimicrobial peptides like human α-defensins (20–22) and cecropins (23). iii) The 34-residue peptide cleavage sites are highly conserved. Considering all 17 prodefensins, cleavage occurs between a Ser and a Leu in 15 of 17 possible cleavage sites, suggesting a serine-specific mechanism. A processing of the propiece from residue 39 and backwards does not make any biological sense because the mature defensin is already achieved. iv) A 34-residue propart is the sole alternative in case of the non-defensin-like peptide CRS4C-4 (Fig. 3B).

We found no qualitative difference between GF and conventional mice with respect to processing of defensins. The peptide pattern was not restricted to the mouse strain NMRI/KI used here because the same peptides were found in C3H/HeJ mice (data not shown). In vitro matrilysin from mouse Paneth cell could cleave prodefensin (24), and it could be responsible for the 39-residue peptide found in conventional mice. However, matrilysin could not be detected in GF mice (25), indicating that other enzymes can be involved in the processing. The first cleavage after residue 34 may be due to an enzyme with a matrilysin-like specificity because the two processing sites after residues 34 and 39 are quite similar (Fig. 3A).

The fact that mice produce defensins independently of microbes (Figs. 1 and 2) whereas bacteria apparently up-regulate matrilysin (25) suggests that matrilysin could speed up the maturation process during bacterial challenge. The defensins from our GF mice must have been synthesized independently of any microbial colonization. It is possible that other factors such as food can stimulate the production and release of antimicrobial factors, either directly or by a hormonal feeding signal. In agreement with our findings, mRNA of defensin 1–3 has been found in GF mice and in the small intestine of the developing mouse (26). It has also been shown that the dramatic increase in Paneth cell number per crypt, which occurs early after birth, did not require stimulation from the microflora (14). However, in isolated crypts, degranulation of the Paneth cells could be induced both by different bacteria and by several bacterial surface products (27). It is possible that on the crypt level, the release of enteric defensins is induced by bacteria and bacterial components, whereas on the organism level, neural or hormonal signaling could add additional levels of regulation of defen-

In conclusion, the processing of mouse defensins includes as a first step cleavage of a Ser-Leu bond, giving a 34-residue propiece peptide. To obtain mature defensins, further processing is needed to remove 4–7 residues. The processing as such may control the level of mature peptides. This, in turn, could depend on functions other than microbicidal, as suggested for defensins 2 and 3 (19). The overall work shows that in vitro data on the production and functions of gene-encoded peptide antibiotics need to be supported or confirmed by in vivo data as obtained here by comparing conventional and germ-free mice.

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