Rhesus θ-defensin 1 (RTD-1) is a unique trisdisulfide, cyclic antimicrobial peptide formed by the ligation of two 9-residue sequences derived from heterodimeric splicing of similar 76-amino acid, α-defensin-related precursors, termed RTD1a and RTD1b (Tang, Y. Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C. J., Ouellette, A. J., and Selsted, M. E. (1999) Science 286, 498–502). The structures of RTD-2 and RTD-3 were predicted to exist if homodimeric splicing of the RTD1a and RTD1b occurs in vivo. Western blotting disclosed the presence of putative θ-defensins, distinct from RTD-1, in leukocyte extracts. Two new θ-defensins, RTD-2 and RTD-3, were purified by reverse-phase high performance liquid chromatography and characterized by amino acid analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy, and comparison to the synthetic standards. RTD-2 and RTD-3 are the predicted homodimeric splicing products of RTD1b and RTD1a, respectively. The cellular abundances of RTD-1, -2, and -3 were 29:1:2, indicating that there is a preference for the heterodimeric ligation that generates RTD-1. RTD-1, -2, and -3 had similar antimicrobial activities against Staphylococcus aureus, Candida albicans, and Cryptococcus neoformans, whereas the activity of RTD-2 against Escherichia coli was 2–3-fold less than those of RTD-1 and RTD-3. Equal amounts of each θ-defensin bound to E. coli cells, indicating that the differences in antibacterial activities are the result of post-binding processes.

Antimicrobial peptides are essential components of the innate immune system (1–4). They play a significant role at the epithelial defense barrier (5–9) and as the antibacterial arsenal in neutrophils and macrophages (10–13). In mammals, defensins and cathelicidins are the two major antimicrobial peptide families (9, 11, 14). Cathelicidins are heterogeneous peptides that share homology in the proregion with cathelin (14). α- and β-defensins are highly conserved trisdisulfide peptides from two genetically distinct families (15, 16). In humans, there are four neutrophil α-defensins, HNP-1–4 (13); two enteric α-defensins, HD-5 and HD-6 (17); and four epithelial β-defensins, hBD-1–4 (18–22). Although hBD-1 is constitutively expressed in epithelia, the expression of hBD-2, -3, and -4 is inducible by inflammatory cytokines (23) or bacterial infection (18, 21).

The characterization of the host defense components of Rhesus macaque granulocytes disclosed two distinct subfamilies of α-defensins (24) and a new trisdisulfide peptide termed rhesus θ-defensin 1 (RTD-1)1 (25). RTD-1 is a macrocyclic 18-amino acid antimicrobial peptide formed by the ligation of two 9-residue sequences derived from similar 76-amino acid, α-defensin-related precursors, termed RTD1a and RTD1b (25). RTD-1 shares some structural similarities with the pig neutrophil protegrins and the horseshoe crab tachyplesins (25–27). The cyclic structure of RTD-1 is an important determinant for microbial potency and resistance to the inhibitory effect of physiologic sodium chloride. The antimicrobial potency of synthetic acyclic RTD-1 was 3-fold lower than that of the native peptide under low salt conditions, and the acyclic peptide was completely inhibited by physiologic NaCl (25). Those studies suggested that the cyclic conformation of RTD-1 confers salt-insensitive microbicidal activity that may be critical for antimicrobial function in the extracellular milieu.

The isolation of RTD-1 revealed the existence of a novel post-translational pathway for the production of head-to-tail cyclized peptides in primates. Based on the heterodimeric splicing model that produces RTD-1 from RTD1a- and RTD1b-derived nonapeptides (25), we hypothesized that two additional θ-defensins, termed RTD-2 and RTD-3, would be produced by the homodimeric splicing of RTD1b and RTD1a, respectively. Here we report the isolation of RTD-2 and RTD-3 from circulating leukocytes, as well as the synthesis and antimicrobial and bacterial binding properties of the three rhesus θ-defensins.

EXPERIMENTAL PROCEDURES

Peptide Synthesis, Disulfide Formation, and Cyclization—Peptide synthesis was performed essentially as described for RTD-1 (25). Peptide sequences corresponding to open-chain versions of RTD-2 and -3 (see Fig. 1) were assembled at 0.2 mmol scale on Fmoc (9-fluorenylmethoxycarbonyl)-Arg(2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonylethanesulfonic acid)-glycylpolyethylene glycol-polystyrene resin using a Milligen 9050 automated synthesizer. Arg, Cys, and Thr side chains were protected with tert-butyl groups, respectively. All amino acids except cysteine were coupled with O-(tert-butyloxycarbonyl)-1-y1-1,3,3-trimetapheryl, hexafluorophosphate/N,N-diisopropylethylamine activation. Cysteine residues were coupled as the pre-formed pentafluorophenyl ester derivative. RTD-2 was assembled with double coupling at every cycle. RTD-3 was assembled with double coupling of Thr and Ile residues.

1 The abbreviations used are: RTD, rhesus θ-defensin; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectroscopy; AU, acid-urea; HOAc, acetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TTBS, Tris-buffered saline plus Tween 20.
Homodimeric θ-Defensins

chain assembly of RTD-2, the peptide resin was cleaved and deprotected by incubation in 20 ml of reagent R (trifluoroacetic acid:thioanisole:1,2-ethanediol; 92:5.3:5; v/v/v, Ref. 25) for 4 h at 22 °C with agitation. Cleavage and deprotection of RTD-3 were performed similarly but using reagent K (trifluoroacetic acid:phenol:water:thioanisole:1,2-ethanediol-v/v/v/v/v). Artifacts from elution fractions were obtained by filtration and extraction with 30% acetic acid/dichloromethane as described for the synthesis of RTD-1 (25).

Linear synthetic RTD-2 and -3 were purified by preparative C18 RP-HPLC on a 25 × 100-mm DeltaPak C18 cartridge (Waters, MA) with a 0.25%-min gradient of water-acetonitrile containing 0.1% trifluoroacetic acid. Aliquots from elution fractions were analyzed by matrix-assisted laser desorption/ionization-time-of-flight mass spectroscopy (MALDI-TOF MS), and those containing reduced/linear peptides were pooled and concentrated 10-fold by centrifugal evaporation. The peptide solutions were diluted to 100–200 μg/ml in 17.4 mM ammonium acetate, pH 8.0, and stirred vigorously in an open container for 18 h at 22 °C. Peptide folding and oxidation were monitored by C18 RP-HPLC and MALDI-TOF MS. The acyclic versions of RTD-2 and -3 were then purified by preparative C18 RP-HPLC as described above. Purity was confirmed by analytical C18 RP-HPLC and acid-urea PAGE on 12.5% polyacrylamide gels (29). For MALDI-TOF MS, peptide solutions were mixed with an equal volume of 10 mg/ml α-cyan-4-hydroxycinnamic acid in 50/50 water-acetonitrile containing 0.1% trifluoroacetic acid and analyzed on a Voyager DE-RP mass spectrometer (PerSeptive Biosystems) (25). Acyclic RTD-2 (10 mg) and RTD-3 (5 mg) were lyophilized, first from 25 mM hydrochloric acid (three times), then from distilled water (twice). Peptide cyclization was carried out by dissolving the lyophilized peptides at 200–300 μg/ml in 0.1% diisopropylethylamine/dimethyl sulfoxide (v/v) containing 60 molar eq of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide and 20 eq of 1-hydroxybenzotriazole. The solutions were sealed under nitrogen and stirred for 18 h at 22 °C. The extent of peptide cyclization was determined by C18 RP-HPLC and MALDI-TOF MS.

Cyclic peptides were purified to homogeneity by C18 RP-HPLC with water-acetonitrile gradients (0.25%/min) containing 0.1% trifluoroacetic acid and characterized by analytical C18 RP-HPLC, AU-PAGE, MALDI-TOF MS, and amino acid analysis (25). The most prominent band on AU-PAGE was excised, homogenized, and immunopositive bands were visualized with Supersignal chemiluminescent substrate (Pierce) on Hyperfilm (Amersham Biosciences, Inc.) using the semi-dry transfer method (30). Replica blots were blocked with 5% nonfat dried milk in TBST (100 mM Tris buffer, pH 7.5, containing 0.9% sodium chloride and 0.1% Tween 20) for 1 h at 22 °C with agitation and incubated with a 1:150 dilution of rabbit anti-RTD-1 (25) or anti-RTD-3 antibody as described previously for the preparation of anti-RTD-1 antibody (25). Briefer, acyclic RTD-3 (3.5 mg) was conjugated to ovalbumin (3.5 mg), dialyzed exhaustively against water, New Zealand White rabbits were repeatedly immunized using standard procedures until the anti-RTD-3 antisem was 1:10,000, as determined by enzyme-linked immunosorbent assay. IgG-enriched preparations were obtained by chromatography on a DEAE Econo-Pac column according to the manufacturer’s protocol (Bio-Rad).

Western Blot Analysis—Five percent acetic acid (HAc) extracts of 1 × 10⁸ leukocytes were resolved on a 12.5% acrylamide polyacrylamide gel and electrophoresed to a 0.22-μm nitrocellulose membrane with an LKB Novablot apparatus (Amersham Biosciences, Inc.) using the semi-dry transfer method (30). Replica blots were blocked with 5% nonfat dried milk in TBTS (100 mM Tris buffer, pH 7.5, containing 0.9% sodium chloride and 0.1% Tween 20) for 1 h at 22 °C with agitation and incubated with a 1:50 dilution (in TBTS) of rabbit anti-RTD-1 (25), anti-RTD-3, or normal rabbit IgG for 1 h. Blots were washed with TBTS (five 10-min washes), and developed with the ABC-Elite kit (Vector Laboratories) as follows: 30-min incubation in biotinylated goat anti-rabbit IgG diluted 1:2,800 in TBTS, 5-min washes (three times), and 30-min incubation in a 1:10 dilution of the avidin-horseradish peroxidase-dase reagent. Immunoactive bands were visualized with Supersignal chemiluminescent reagent substrate (Pierce) on Hyperfilm (Amersham Biosciences, Inc.). Purification of Natural RTD-1—Peripheral blood leukocytes (>90% PMN) were obtained from four rhesus monkeys. Approximately 1–1.8 × 10⁸ leukocytes/animal were extracted with 5% HAc as described previously (24, 25). Pooled acid extracts of 1–10 × 10⁹ cell eq were fractionated on a 4.6 × 250-mm Vydac C18 column using a linear acetonitrile gradient (30–90% acetonitrile in 30 min) containing 0.1% trifluoroacetic acid or 0.1% phosphoric acid (H₃PO₄). Eluant fractions were analyzed by MALDI-TOF MS for peptides with molecular masses of RTD-1–3. Three θ-defensins were purified to homogeneity by successive rounds of RP-HPLC using the same solvent gradient but alternating between 0.1% trifluoroacetic acid and 0.1% H₃PO₄ as the ion pairing reagent. Purified RTD-1–3 were characterized by MALDI-TOF MS, amino acid analysis, and AU-PAGE. Cysteine content was determined by comparing the mass of the native peptides with those obtained following reduction of disulfides with 1,4-dithiothreitol and alkylation with iodoacetamide (31). The amino acid compositions of RTD-1–3 were determined on an AE-6000 amino acid analyzer (Applied Biosystems). The antimicrobial activities of synthetic RTD-1–3 against bacteria (Staphylococcus aureus 502a and Escherichia coli ML35) and fungi (Candida albicans 16820 and Cryptococcus neoformans 271A) were determined in an agar diffusion assay as described previously (33). Briefly, 10-μl wells were bored in a 9-cm² plate of agarose, buffered with 10 mM PIPES, pH 7.4, containing 5 mM glucose, and seeded with 1 × 10⁷ colony-forming units/ml. Five-μl aliquots of each peptide, dissolved in 0.01% HAc at 10–30 μg/ml, were added to each well. After incubation at 37 °C for 2 h, the seeded agar was overlaid with molten agarose containing 6% trypticase soy broth (for bacteria) or Sabouraud dextrose agar (for fungi). Plates were incubated at 37 °C for 18–24 h, and antimicrobial activity was determined by measuring the diameter of clearing around each well.

Acyclic peptide was determined by incubating 2 × 10⁹ colony-forming units/ml with peptides (0.5–12 μg/ml) in 50 μl of low salt dextran, 10 mM PIPES buffer containing 5 mM glucose, pH 7.4, or the same diluent supplemented with 25–150 mM NaCl. After 2 h of incubation at 37 °C, the cell suspensions were diluted 1:50 with 10 mM sodium phosphate buffer, pH 7.4, and exponentially spread with an Autoplate 400 (Spiral Biotech) onto trypticase soy agar (bacteria) or Sabouraud dextrose agar (fungi). After incubation at 37 °C for 18–48 h, colonies were counted and cell survival was expressed as colony-forming units/ml.

Binding of RTD-1–3 to E. coli ML35 was evaluated by incubating 2 × 10⁶ log-phase bacteria with increasing peptide concentrations (0.5–8 μg/ml final) in 1 ml of 10 mM PIPES, pH 7.4, containing 5 mM glucose for 2 h at 37 °C (25). The incubations were centrifuged at 20,000 g at 25,000 × g for 10 min at 22 °C, and supernatant samples were analyzed for RTD content by RP-HPLC. Binding of each to RTD-1 was determined by subtraction of the quantity of supernatant peptide from total added to each tube, and comparing this to control incubations lacking bacteria.

RESULTS

Peptide Synthesis, Disulfide Formation, and Cyclization—RTD-2 and -3 are cyclic analogs of RTD-1 predicted to be produced by homodimeric splicing of nonapeptides from RTD1b and RTD1a, respectively (Fig. 1). Linear RTD-2 and -3 were synthesized and purified by preparative RP-HPLC. Disulfide bond formation proceeded efficiently in room air, giving >90% yield of monomeric, tridisulfide peptide as determined by quantitative RP-HPLC and MALDI-TOF MS. The yields of the subsequent peptide cyclization steps were 92% for RTD-2 and 64% for RTD-3. The cyclic peptides were purified by C18 RP-HPLC and characterized by AU-PAGE, amino acid analysis, and MALDI-TOF MS. RTD-2 (9.2 mg) and RTD-3 (3.2 mg) preparations were more than 98% pure, and were indistinguishable from the natural peptides (see below).

Isolation of Natural RTD-1—Synthetic RTD-1, -2, and -3 had unique λₚ values on acid-urea PAGE because of their differing arginine contents (Fig. 2). Acid extracts of rhesus macaque leukocytes contained a band that co-migrated with synthetic RTD-1 on AU-PAGE and Western blots. RTD-1 and two additional immunopositive bands that migrated with RTD-2 and RTD-3 synthetic standards were detected in leukocyte extracts with anti-RTD-1 and anti-RTD-3 antibodies (Fig. 2). These data strongly suggested the presence of RTD-2 and RTD-3 in leukocyte extracts.

RTD-1–3 were isolated from leukocyte extracts by RP-HPLC (Fig. 3). Peptides with masses of RTD-1, -2, and -3 were detected in three peaks following the initial chromatographic step (Fig. 3A), and the RP-HPLC elution times precisely matched those determined for synthetic peptides (Fig. 3B), and the RP-HPLC elution times precisely matched those determined for synthetic peptides.
those of the respective synthetic peptides. Each θ-defensin was purified to homogeneity (Fig. 3B), and their identities were confirmed as described below.

Characterization of RTD-2 and -3—Automated Edman degradation of 20–50 pmol of purified RTD-2 and RTD-3 yielded no amino acid signal, consistent with the θ-defensin cyclic structure. The molecular masses of natural RTD-2 and RTD-3, determined by MALDI-TOF MS, matched the calculated values of the predicted sequences (Fig. 1). The cysteine content of purified RTD-1–3 was determined by comparing the molecular masses of native peptides with those that had been reduced and alkylated. Carboxamidomethylated RTD-1, -2, and -3 had molecular masses of 2430.5 atomic mass units (2430.7 theoretical), 2436.9 atomic mass units (2437.7 theoretical), and 2424.5 atomic mass units (2423.6 theoretical), respectively, consistent with the complete alkylation of 6 cysteine residues in each θ-defensin (Table I).

The compositions of natural RTD-2 and -3, determined by amino acid analysis of peptide hydrolysates (Table I), were consistent with those of the corresponding structures shown in Fig. 1 (B and C), and that of purified RTD-1, which was as reported previously (25). In a previous study, synthetic RTD-1 was biochemically and functionally equivalent to the natural peptide (25). Synthetic RTD-2 and -3 were also indistinguishable from the natural isolates by amino acid analysis, MALDI-TOF MS, AU-PAGE, and analytical RP-HPLC (Fig. 4).

The amount of each θ-defensin isolated from acid extracts of rhesus leukocytes was determined by quantitative amino acid analysis. The cellular abundance of θ-defensins in extracts of 5.8 × 10^8 cell eq was 107 μg (51.4 nmol) of RTD-1, 3.8 μg (1.8 nmol) of RTD-2, and 8.8 μg (4.2 nmol) of RTD-3, giving relative cellular abundances of 29:1:2 (RTD-1:RTD-2:RTD-3). These data indicate that 10-fold more heterodimeric RTD-1 is present in cells than the homodimeric homologs.

Antimicrobial Activities of RTD-1–3—An agar diffusion assay was utilized to assess the combined microbicidal and microbistatic activities of RTD-1–3 against Staphylococcus aureus 502a, Escherichia coli ML35, and yeast forms of Candida albicans 16820 and Cryptococcus neoformans 271A. The antimicrobial activities of the three θ-defensins were equivalent against S. aureus, C. albicans, and C. neoformans. RTD-2 was 2–3-fold less active than RTD-1 and RTD-3 against E. coli (Fig. 5). The
organisms in the presence of 0–microbicidal activities of RTD-1 is relatively unaffected by physiologic NaCl (25). The S. aureus shown). The activities of affected by NaCl at all salt concentrations tested (data not covered in animals, disclosing the capacity of animal cells to possess antimicrobial activities suggests that these peptides may have a role in plant defense (40, 41).

RTD-1 was the first macrocyclic antimicrobial peptide discovered in animals, disclosing the capacity of animal cells to produce this circular motif (25). The fact that RTD-1 is produced by the heterodimeric ligation of two nonidentical nonapeptides encoded by distinct genes suggested that homodimers might be formed similarly. The isolation of RTD-2 and RTD-3 confirmed this hypothesis. Moreover, the biosyn-

**Discussion**

Macrocyclic peptides composed entirely of L-amino acids are relatively rare biomolecules, nearly all known examples of which have been isolated from plants. More than 30 macrocyclic peptides, collectively termed cycloptides, have been identified in plants of the Rubiaceae and Violaceae families (36). The mature active peptides typically contain 30 amino acids including 6 cysteines connected in a 1–6 motif. The structures of two cyclotides (circulin A and cycloviolacin O1), determined by NMR, contain several β-strands constrained by a cysteine knot (36, 37). Peptide folding kinetics of acyclic precursors (38, 39). The observation that some cyclotides possess antimicrobial activities suggests that these peptides may have a role in plant defense (40, 41).

**TABLE I**

| Amino acid compositions of rhesus θ-defensins |
|---------------------------------------------|
| RTD-1 | RTD-2 | RTD-3 |
|-------|-------|-------|
| Arg   | 4.84 (5) | 5.63 (6) | 3.78 (4) |
| Cys   | 6.00 (6) | 6.00 (6) | 6.00 (6) |
| Gly   | 2.03 (2) | 2.20 (2) | 2.19 (2) |
| Ile   | 0.96 (1) | 0 (0)       | 2.19 (2) |
| Leu   | 1.01 (1) | 2.00 (2) | 0 (0)   |
| Phe   | 1.05 (1) | 0 (0)       | 2.00 (2) |
| Thr   | 1.00 (1) | 0 (0)       | 1.94 (2) |
| Val   | 0.98 (1) | 1.92 (2) | 0 (0)   |
| Molecular mass |       |       |       |
| Cyclic, oxidized | 2082.7a | 2089.7b | 2075.6c |
| Experimental | 2083.0a | 2087.9b | 2076.0c |
| Linear, reduced | 2106.7a | 2113.7b | 2099.6c |
| Difference | 24a | 24b | 24c |

* Calculated based on cyclic structures shown in Fig. 1 (B and C).
* Determined by MALDI-TOF mass spectroscopy.

**Fig. 3. Purification of RTD-1–3.** A, acid extracts of 4 × 10⁷ leukocytes were chromatographed on a C18 reverse-phase column using a 0.5%/min water-acetonitrile gradient containing 0.1% trifluoroacetic acid. Numbered peaks contained peptides with molecular masses shown, including masses consistent with RTD-1, -2, and -3. B, purified RTD-1–3 were chromatographed on a C18 column using a 1%/min water-acetonitrile gradient containing 0.1% trifluoroacetic acid.

**Fig. 4. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 5. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 6. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 7. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 8. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 9. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 10. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 11. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 12. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.

**Fig. 13. Peptide lethality.** The potency of synthetic RTD-1, -2, and -3 was measured against the four test organisms.
thesis of three unique \( \theta \)-defensins suggests that the cellular machinery responsible for peptide cyclization may be involved in post-translational modification of other gene products (25).

The cyclic structures of RTD-1–3 endow the peptides with resistance to exoproteinases, and this may be advantageous in a protease-rich inflammatory milieu. Furthermore, peptide cyclization confers other properties to RTD-1 (25) that are absent in an acyclic analog, because acyclic RTD-1 is substantially less active than the native peptide against \( S. \) aureus and \( E. \) coli (25). RTD-1–3, but not the respective acyclic analogs, maintain their staphylocidal and colicidal activities in the presence of physiologic sodium chloride (Ref. 25 and data not shown).

The relative yields of RTD-1, -2, and -3 obtained from leukocyte extracts indicated that RTD-1 is 10-fold more abundant than RTD-2 and -3 combined. This suggests a strong preference for production of RTD-1 by heterodimeric splicing of the RTD-1 precursors, RTD1a and RTD1b. We speculate that distinct elements in the two precursors may direct the assembly of heterodimeric intermediates prior to peptide splicing (25).

Despite differences in cationicity, RTD-1 (+5), RTD-2 (+6), and RTD-3 (+4) possess similar antimicrobial potencies against four organisms tested in this study, and were nearly identical against \( S. \) aureus, \( C. \) albicans, and \( C. \) neoformans. The most cationic peptide, RTD-2, was slightly less active against \( E. \) coli than RTD-1 and RTD-3. This was somewhat surprising because increased cationicity typically correlates with greater antimicrobial activities and increased spectrum of
Homodimeric θ-Defensins

Addendum—As the current studies were being prepared for publication, we became aware of a report by Leonova et al. (44), who described the isolation of RTD-1 from rhesus macaque bone marrow, the structures of which are identical to those reported here. Further, the solution structure of synthetic RTD-1 was recently reported by Trabi et al. (45), fundamentally confirming the model proposed previously (25).

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