Antimicrobial peptides are evolutionarily ancient elements of innate immunity (1). Mammalian defensins, comprising genetically distinct α and β subfamilies, are cationic, trisulfide-containing antimicrobial peptides in granules of neutrophils and monocytes. The peptide, termed rhesus theta defensin-1 (RTD-1), is microbicidal for bacteria and fungi at low micromolar concentrations. Antibacterial activity of the cyclic peptide was threefold greater than that of an open-chain analog, and the cyclic conformation was required for antimicrobial activity in the presence of 150 millimolar sodium chloride. Biosynthesis of RTD-1 involves the head-to-tail ligation of two α-defensin–related nonapeptides, requiring the formation of two new peptide bonds. Thus, host defense cells possess mechanisms for synthesis and granular packaging of macrocyclic antibiotic peptides that are components of the phagocyte antimicrobial armamentarium.

Analysis of rhesus macaque leukocytes disclosed the presence of an 18-residue macrocyclic, trisulfide antibiotic peptide in granules of neutrophils and monocytes. The peptide, termed rhesus theta defensin-1 (RTD-1), is microbicidal for bacteria and fungi at low micromolar concentrations. Antibacterial activity of the cyclic peptide was threefold greater than that of an open-chain analog, and the cyclic conformation was required for antimicrobial activity in the presence of 150 millimolar sodium chloride. Biosynthesis of RTD-1 involves the head-to-tail ligation of two α-defensin–related nonapeptides, requiring the formation of two new peptide bonds. Thus, host defense cells possess mechanisms for synthesis and granular packaging of macrocyclic antibiotic peptides that are components of the phagocyte antimicrobial armamentarium.

RTD-1 was isolated from rhesus macaque leukocytes [91% polymorphonuclear (PMN)] by reversed-phase (RP) high-performance liquid chromatography (HPLC) of a whole-cell extract enriched for α-defensins (Fig. 1) (5).

Fig. 1. Purification of RTD-1. (A) RP-HPLC of peripheral blood leukocyte extracts. An α-defensin-enriched extract of 6 × 10^6 leukocytes (91% PMNs) (8) was fractionated by RP-HPLC on a 0.46 × 25 cm C-18 column equilibrated in 0.1% aqueous TFA and developed with a linear acetonitrile gradient (dotted line). RTD-1 eluted in the arrow-marked peak. (B) Analytical RP-HPLC of purified RTD-1. The purity of RTD-1 was assessed by RP-HPLC of RTD-1 obtained from the peak (arrow) in (A) on an analytical C-18 column equilibrated in aqueous 0.13% HFBA and developed with a linear (20 to 60%) acetonitrile gradient in 20 min. (C) Acid-urea PAGE. Samples of 30% acetic acid granulocyte extract (2 × 10^6 cell equivalents; lane 1), the subsequent methanol-water extract (1 × 10^6 cell equivalents; lane 2), and 0.4 μg of RTD-1 were resolved on a 12.5% acid-urea polyacrylamide gel and stained with Coomassie blue.
To distinguish between the eight possible disulfide pairings in the 17-mer, the oligopeptide was digested with thermolysin and the resulting fragments were analyzed by MALDI-TOF MS (9). The $m/z$ values of the thermolysin fragments were consistent with only one cystine motif (Fig. 2C), revealing that the peptide ring is stabilized by three disulfides in a trimer threefold symmetry–like array that crosslinks two hypothetical $\beta$-strands connected by turns at both ends (Fig. 2, D and E). Schematically, RTD-1 resembles the Greek letter theta (Fig. 2D), hence the term $\theta$-defensin to describe this molecular motif. The peptide is highly cationic, possessing a net charge of $\approx 5 \text{ at pH } 7$ (calculated pI 12), and the dense cystine motif is distinct from that determined for $\alpha$- or $\beta$-defensins (14).

The structure and activities of RTD-1 were confirmed by the preparation and characterization of synthetic RTD-1. The purified synthetic peptide was identical to natural RTD-1 as determined by RP-HPLC, acid-base linearization, MALDI-TOF, circular dichroism spectroscopy, disulfide motif, and quantitative microbicidal activity (supplemental figures 1 and 2 (15)). Together these data confirm the structure and biological activity of native RTD-1.

Searches for amino acid sequence similarity to RTD-1 were carried out using all 18 possible linearized peptides as query sequences (16). Taking into consideration the cysteine spacing and disulfide connectivities of RTD-1, the most similar polypeptide sequence identified was that of protegrin-3 (PG-3), an antimicrobial peptide from pig neutrophils (17). Protegrins are 17- to 18–amino acid, bis-disulfide-containing peptides that are members of the cathelicidin family of antimicrobial peptides (18). Like protegrins, RTD-1 is predicted to be predominantly composed of two disulfide-stabilized $\beta$-strands connected by turns. A model of RTD-1, derived from molecular dynamics simulations (Fig. 2E) (19), is remarkably similar to the solution structure of protegrin-1 (PG-1), an isoform of PG-3. Despite this similarity, cloning experiments revealed that RTD-1 is not a rhesus cathelicidin.

The occurrence of RTD-1 in primate phagocytes discloses a structural motif in animals resembling a group of macrocyclic peptides previously characterized in plants. Like RTD-1, cyclic peptides isolated from plants of the Rutaceae family molecules possess three intramolecular disulfides (20). Two of these peptides are reported to be antiviral against HIV-1 (21). The plant peptides differ from RTD-1 in their size (29 to 31 versus 18 amino acids, respectively) and they contain a cystine motif that is characterized by “overlapping” disulfides that produce a cystine knot (22). Thus far, the genes encoding these plant peptides have not been reported, nor have mechanisms been proposed for the formation of the cyclic backbone. The only other macrocyclic peptides that we are aware of lack disulfides. One, AS-48, is a plasmid-encoded peptide expressed by Enterococcus faecalis (23); the second is J25, a microcin from E. coli (24).

Cloning of RTD-1 precursors. To elucidate the RTD-1 biosynthetic pathway, we sought to characterize the peptide precursor by analysis of one or more of the corresponding cDNAs from rhesus macaque bone marrow mRNA. Because the NH$_2$-terminus of RTD-1 could not be predicted from its cyclic sequence, we conducted 3’ RACE (rapid amplification of cDNA ends) experiments using degenerate oligonucleotide primers corresponding to six to seven amino acid segments of the peptide sequence (25). These amplifications consistently yielded two sequences that separately corresponded to different portions of RTD-1: R$^8$-R$^9$-G$^{10}$-V$^{11}$-C$^{12}$-[Q-L-L-stop] and T$^{17}$-R$^{18}$-G$^{19}$-F$^{20}$-C$^{21}$-[R-L-L-stop] (Fig. 2D) (25). The 3’ RACE products were then used to probe a rhesus macaque bone marrow cDNA library (26). Fifteen positive clones were isolated and sequenced, revealing two highly similar cDNAs (92% identity) termed RTD1a and RTD1b (Genbank accession numbers AF191100 and AF191101, respectively). Surprisingly, none of the clones analyzed contained a sequence that coded for all 18 amino acids in RTD-1.

RTD1a and RTD1b each encode 76–amino acid pre-propeptides containing a 20-residue signal peptide and a 44–amino acid prosegment, which are identical in size and

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**Figure 2.** Peptide backbone structure of RTD-1. (A) The amino acid sequence of the peptide chain was determined by Edman sequencing or MALDI-TOF MS, or both, of fragments produced by methanolic-HCl treatment or digestion with chymotrypsin (CT) and trypsin (T). Disulfides were reduced with dithiothreitol and alkylated with 4-vinyl pyridine before digestion so that cysteines were analyzed as 5-pyridylethyl cysteine. Residues in parentheses were assigned based on MALDI-TOF MS data. Calculated MALDI-TOF MS values are in parentheses. (B) Amino acid sequence of the RTD-1 peptide backbone. (C) Disulfide analysis of RTD-1. A tridisulfide-containing 17-residue oligopeptide generated by trypsin digestion was purified by RP-HPLC and digested further with thermolysin (13). MS analyses (calculated values in parentheses) of the digest disclosed thermolysin cleavage at Cys-14/Ile-15 and at Cys-5/Leu-6 (arrows), producing four major thermolytic fragments (Th-1 to Th-4). The masses of all fragments from several digests were uniquely consistent with the disulfide assignments shown. (D) Schematic of the covalent structure of RTD-1. The highlighted amino acids correspond to the two 3’ RACE products obtained (25), and the color scheme indicates the precursors (Fig. 3, A and C) from which they are derived. (E) A theoretical model of RTD-1 obtained by molecular dynamics and energy minimization in water is shown (19).
similar in sequence to homologous regions of α-defensin precursors (Fig. 3A) (27). Alignment (16) of the RTD1a- and RTD1b-derived precursor sequences with that of HNP-4, the most similar α-defensin, disclosed substantial similarities at the amino acid level (Fig. 3A). However, the covalent structures of mature RTD-1 and HNP-4 (30 amino acids) are markedly dissimilar (28). Furthermore, compared to α-defensins, RTD-1 precursors have truncated COOH-terminal segments, encoding only 12 residues after the putative junction between the prosegment and a COOH-terminal dedcapeptide (Fig. 3A).

Genomic clones RTD1.1 and RTD1.2, corresponding to the RTD1a and RTD1b cDNAs, respectively, were isolated from a rhesus macaque genomic library (29), confirming that the cDNAs derive from distinct transcriptional units. RTD1.1 and RTD1.2 are 93% identical, and the three-exon, two-intron gene structure and organization that they share are very similar to those of the myeloid α-defensin genes characterized previously in humans, rabbits, and guinea pigs (Fig. 3B) (30). The close relationship between the RTD-1 and α-defensin genes is also shown by their 88% nucleotide sequence identity with a human α-defensin–related pseudogene (accession U10267). Interestingly, one of the stop codons in this pseudogene corresponds exactly to the position of the stop codons that truncate RTD-1 coding sequences (Fig. 3A).

Comparison of the RTD-1 covalent structure with that of the deduced RTD1a and RTD1b translation products revealed that the cyclic peptide is composed of two nine-residue peptides that are derived separately from the RTD1a and RTD1b precursors. RTD-1 peptide residues 13 to 18 and 1 to 3 correspond to amino acids 65 to 73 from RTD1a, and RTD-1 residues 4 to 12 correspond to RTD1b amino acids 65 to 73 (Fig. 3, A and C). The transformation of the two linear precursors into cyclic RTD-1 requires that two head-to-tail ligation reactions join the constituent nonapeptides by posttranslational reactions not previously recognized (Fig. 3C).

Expression of RTD-1 in myelopoietic elements. The anatomic distribution of RTD-1 gene expression in rhesus macaque tissues was determined by Northern blotting using a probe specific for RTD-1 mRNAs (31). Of the 18 organs analyzed, only bone marrow was positive for RTD-1 mRNA (Fig. 4A). The leukocytic lineages in which RTD-1 is expressed were identified by immunohistochemical staining with an anti–RTD-1 antibody (32). Dot blot analysis demonstrated that anti–RTD-1 antiseraum reacted with natural and synthetic RTD-1 and the oxidized acyclic version of RTD-1, but did not recognize any of the previously characterized α-defensins (HNP-1 through HNP-4) expressed by human leukocytes nor any of the rhesus leukocyte α-defensins. Immunostaining ofuffy coat leukocytes demonstrated strong, punctate staining in neutrophil cytoplasm, similar to the immunolocalization of neutrophil α-defensins, which are stored in azurophil granules (Fig. 4, B and C). Although less reactive than neutrophils, monocytes were also immunopositive, but lymphocytes and eosinophils were negative. Thus, RTD-1 is expressed in the two major phagocytic cells of the blood.

RTD-1 gene expression and peptide accumulation begin early during granulocyte myelopoiesis. Staining of bone marrow cells with anti–RTD-1 antibody demonstrated immunoreactivity in the cytoplasm of immature myeloid elements (promyelocytes, myelocytes) and in mature neutrophils and monocytes (Fig. 4D). This pattern of expression during myeloid differentiation and the punctate pattern of immunostaining are consistent with the packaging of RTD-1 in azurophil granules.

Microbicidal activity. The in vitro antimicrobial properties of RTD-1 were evaluated in microbicidal assays against a panel of bacterial and fungal test organisms. The broad spectrum of RTD-1 antimicrobial activity was demonstrated in microbicidal assays against Gram-positive bacteria (S. aureus, Listeria monocytogenes), Gram-negative bacteria (E. coli ML 35, Salmonella typhimurium), and fungi (Candida albicans and Cryptococcus neoformans). The viability of each organism was reduced by more than 99% after 2-hour incubations with 2 to 4 μg/ml RTD-1, and higher peptide concentrations (up to 20 μg/ml) effected killing to levels below the detection limit of the assay (Fig. 5, A through C).

The mechanistic significance of the RTD-1
cyclization was investigated by comparing the antibacterial activities of the cyclic peptide to that of the trisdilufide-containing acyclic analog from which it was produced [supplemental figure 1(15)]. The peptides were tested for activity against *S. aureus* and *E. coli* in an agar diffusion antimicrobial assay (6). RTD-1 was three times as active as the acyclic analog against both organisms, indicating that cyclization confers a substantial increase in antimicrobial potency (Fig. 5, D and E).

Previous studies have demonstrated that α- and β-defensin–mediated microbicidal activity is antagonized by increasing the ionic strength of the incubation medium (33). In this context, it has been proposed that salt sensitivity of airway β-defensins underlies the susceptibility of cystic fibrosis patients to pulmonary infections (34). Therefore, the effect of ionic strength on natural and synthetic RTD-1 staphylocidal activity was determined. Sodium chloride concentrations as high as 150 mM had little effect on the staphylocidal activity of natural or synthetic RTD-1 (Fig. 5F), but the acyclic form of RTD-1 was inhibited nearly completely by 125 mM NaCl. These data demonstrate that the cyclized conformation confers salt-insensitivity to RTD-1. We speculate that under conditions of low (Fig. 5, D and E) and high (Fig. 5F) ionic strength, the cyclic conformer more efficiently binds to and inserts into the target cell envelope than does the acyclic peptide, which possesses additional charge at both termini.

The broad spectrum of RTD-1 antimicrobial activity and its expression in phagocytes raises questions about the evolutionary forces that might have selected for the production of
cyclization of other polypeptide precursors. Inflammatory exudates, and the lack of NH2- and COOH-termini eliminates its proteolytic removal of the prosegments and the formation of the one interchain disulfide rearrangement may occur in this process. The cyclic structure of RTD-1 demonstrates that the two hairpin halves of RTD-1 so connected by three disulfides (Fig. 2C). A 50-pmol sample of the 17-amino acid fragment was digested at 37°C for 10 min with 0.1 mg TPCK trypsin or TLCK α-chymotrypsin in 50 μl of 0.1% ammonium bicarbonate, pH 8.0. Peptide fragments were purified by RP-HPLC and characterized by amino acid analysis, MALDI-TOF MS, and automated sequencing.

2. Peptide (1 nmol) was dissolved in 24 μl of 1 M methanolic HCl for 48 hours at room temperature. Edman sequencing of two separate reaction mixtures disclosed the sequence shown in Fig. 2A.
3. A 2-nmol sample of 5-pyridylthylated peptide was digested at 37°C for 10 min with 0.1 mg TPCK trypsin or TLCK α-chymotrypsin in 50 μl of 0.1% ammonium bicarbonate, pH 8.0. Peptide fragments were purified by RP-HPLC and characterized by amino acid analysis, MALDI-TOF MS, and automated sequencing.
4. A 2.5-nmol sample of RTD-1 was digested at 37°C for 16 hours with 0.5 μg TPCK trypsin in 50 μl of 0.1 M pyridine acetate. 6.4 μl of this mixture was applied to a reversed-phase HPLC column and fractions were collected for MS analysis.
6. Peptide (1 nmol) was dissolved in 24 μl of 1 M methanolic HCl for 48 hours at room temperature. Edman sequencing of two separate reaction mixtures disclosed the sequence shown in Fig. 2A.
7. A 2-nmol sample of 5-pyridylthylated peptide was digested at 37°C for 10 min with 0.1 mg TPCK trypsin or TLCK α-chymotrypsin in 50 μl of 0.1% ammonium bicarbonate, pH 8.0. Peptide fragments were purified by RP-HPLC and characterized by amino acid analysis, MALDI-TOF MS, and automated sequencing.
8. The cyclic structure of RTD-1 demonstrates that the two hairpin halves of RTD-1 so connected by three disulfides (Fig. 2C). A 50-pmol sample of the 17-amino acid fragment was digested at 37°C for 10 min with 0.1 mg TPCK trypsin or TLCK α-chymotrypsin in 50 μl of 0.1% ammonium bicarbonate, pH 8.0. Peptide fragments were purified by RP-HPLC and characterized by amino acid analysis, MALDI-TOF MS, and automated sequencing.
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10. Peptide (1 nmol) was dissolved in 24 μl of 1 M methanolic HCl for 48 hours at room temperature. Edman sequencing of two separate reaction mixtures disclosed the sequence shown in Fig. 2A.
11. Peptide (1 nmol) was dissolved in 24 μl of 1 M methanolic HCl for 48 hours at room temperature. Edman sequencing of two separate reaction mixtures disclosed the sequence shown in Fig. 2A.
12. Peptide (1 nmol) was dissolved in 24 μl of 1 M methanolic HCl for 48 hours at room temperature. Edman sequencing of two separate reaction mixtures disclosed the sequence shown in Fig. 2A.