Structural Characterization of the Inflammatory Moiety of a Variable Major Lipoprotein of *Borrelia recurrentis*

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Louse-borne relapsing fever, caused by *Borrelia recurrentis*, provides one of the best documented examples of the causative role of tumor necrosis factor (TNF) in the pathology of severe infection in humans. We have identified the principal TNF-inducing factor of *B. recurrentis* as a variable major lipoprotein (Vmp). Here we report the complete gene sequence of Vmp, including its lipoprotein leader sequence. Using metabolically labeled forms of the native Vmp we confirm that the TNF inducing properties are associated with the lipid portion of the molecule. Quadrupole orthogonal time of flight mass spectrometry unequivocally locates the lipic moiety at the NH₂-terminal cysteine of the native polypeptide, and indicates the existence of three forms which are consistent with the structures C₁₆:₀, C₁₆:₀, C₁₆:₀ glyceryl cysteine; C₁₈:₁, C₁₆:₀, C₁₆:₀ glyceryl cysteine; and C₁₈:₀, C₁₆:₀, C₁₆:₀ glyceryl cysteine. These data provide the first direct evidence that the TNF inducing lipid modification of native *Borrelia* lipoproteins is a structural homologue of the murein lipoprotein of *Escherichia coli*.

Lipoproteins have been implicated as the principal mediators of the inflammatory response that occurs in the human host during a spirochetal infection. In vitro, it has been clearly established that outer surface lipoproteins of spirochetes causing Lyme disease and syphilis induce the production of inflammatory mediators from a variety of cell types (1–11). Recently, we identified a variable major lipoprotein (Vmp) as the principal TNF inducing stimulus of *Borrelia recurrentis*, the causative agent of louse borne relapsing fever (12). The TNF-inducing stimulus of *B. recurrentis* is of particular interest since this is the only human infectious disease in which anti-TNF therapy has been conclusively demonstrated to have a beneficial role (13).

Several studies have demonstrated that lipid modification is essential for the potent biological activities of spirochetal lipoproteins (3, 12, 14, 15). The structure of the lipid modification of a native spirochetal lipoprotein has not been elucidated but features of lipoproteins from *Borrelia burgdorferi* provide insight into a possible structure. Metabolic labeling studies with palmitic acid and the presence of a consensus lipoprotein leader sequence indicate a lipid modification similar to that of *Escherichia coli* murein lipoprotein (16). However, indirect evidence from functional comparisons suggests that the lipid modifications of *B. burgdorferi* and *E. coli* lipoproteins may differ. First, *B. burgdorferi* lipoproteins have greater potency than *E. coli* murein lipoprotein (2, 3, 5, 17, 18); and second, *B. burgdorferi* lipoproteins are considerably more potent than corresponding lipopeptides synthesized with the *E. coli* type of lipid modification having three palmitic acid residues (Pam3Cys) (3).

The lack of direct evidence concerning the native lipid modifications of spirochetal lipoproteins, and the established clinical importance of TNF induction in louse borne relapsing fever, led us to investigate the biochemical structure of the Vmp of *B. recurrentis*.

**EXPERIMENTAL PROCEDURES**

Cloning and Sequencing of vmp A1—DNA isolated from *B. recurrentis* (19) was digested with HindIII and cloned into pBluescript. The resulting library was screened with a partial vmp A1 gene PCR product (12) that was radiolabeled using a random priming kit (Megaprime kit, Amersham Pharmacia Biotech). Positive clones were sequenced using an ABI 377 sequencer using BigDye terminator chemistry (PerkinElmer). DNA sequences were assembled and analyzed using a software package from the Wisconsin Genetics Computer Group, Madison, WI.

**Purification of Native Vmp A1—** *B. recurrentis* isolate A1 was grown in BSK II medium plus 6% rabbit serum. Cells from a 1-liter culture were harvested at late logarithmic phase and washed twice with HEPES-buffered saline (HBS; 25 mM HEPES, pH 7.5, 150 mM NaCl) and sonicated in 1 ml of HBS. The lysate was centrifuged for 15 min at 10,000 × g, and the pellet was extracted twice with HBS and once with methanol:chloroform:water (8:4:3). The pellet was solubilized with 0.1% SDS in HBS, and further purified and desalted by reverse phase (RP)-HPLC (12). This preparation was of greater than 95% purity.

**Assay of TNF Induction—** To assay TNF induction we used the human monocyte cell line MonoMac6 (MM6, German Collection of Microorganisms and Cell cultures) (20). Cells were grown in RPMI 1640 medium containing 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 1 × non-essential amino acids, 9 µg/ml insulin, 1 mM oxaloacetic acid, and 10% fetal calf serum. Prior to assay cells were plated out in 96-well flat bottom plates at 2 × 10⁶ cells/ml (100 µl/well) in the presence of 100 ng/ml phorbol 12-myristate 13-acetate and incubated for 1 h. Test stimuli were then added to the cells for 16 h and supernatants were assayed for TNF by an enzyme-linked immunosorbent assay as described previously (21).

**Purification of Native TNF Inducing Lipopeptide Derived from Vmp A1—** A 15-ml culture of *B. recurrentis* isolate A1 was grown from early to late logarithmic phase in the presence of 4 mM of 9,10-2'-Hpalmitic acid. The cells were harvested and washed three times with HBS,
sonicated, and extracted with HBS and methanol:chloroform:water as above and then extracted with 0.1% SDS in HBS. The SDS extract was further purified by preparative electrophoresis (12). TNF inducing fractions containing Vmp were concentrated by ultrafiltration and precipitated with 10% trichloroacetic acid. The precipitated material was resuspended in 2 M urea, 0.1 M ammonium bicarbonate buffer, and digested for 16 h at 37 °C with 2% (w/w) trypsin. The digest was dried under vacuum, and redissolved in 0.2 ml of 0.1% trifluoroacetic acid in water to which 1.2 ml of chloroform:methanol (2:1) was added. The chloroform:methanol phase was removed, dried under vacuum, and redissolved in hexane, 2-propanol, 0.1% trifluoroacetic acid in water (3:4:0.25, solvent A). The recovery of radioactivity at each stage was redissolved in 0.2 ml of 0.1% trifluoroacetic acid in chloroform:methanol phase was removed, dried under vacuum, and redissolved in 0.1% SDS in HBS. To generate larger amounts of lipopeptide for mass spectrometry the process was repeated for a 1-liter culture without palmitic acid.

**RESULTS**

**Characterization of Vmp A1 Lipoprotein**—The protein component of the lipoprotein was investigated by cloning and sequencing the gene encoding Vmp A1. A genomic library was screened using a T75-base pair polymerase chain reaction product that partially encodes vmp A1 (12). An open reading frame encoding a protein of 363 amino acids was identified in positive clones (Fig. 1A). This sequence contains a consensus prokaryotic membrane lipoprotein leader sequence from residue 1 to 26. Thus, the putative mature protein contains 338 amino acids and has a predicted molecular mass of 34,287 daltons. The consensus leader sequence from residue 1 to 26 is underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number AJ237608. The consensus prokaryotic lipoprotein leader sequence from residue 1 to 26 is underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number AJ237608.

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The data transform to give a minimum molecular mass of 35,077 daltons for Vmp A1, that is 790 daltons higher than that predicted from the gene sequence, thus confirming the presence of one or more post-translational modifications on Vmp A1. Two other proteins of masses 35,101 and 35,120 daltons were detected; these may be sodium adducts or other forms of Vmp A1 having modifications of greater molecular mass. The peak located at about 35,025 daltons may be a cone-voltage induced loss from the molecular ions or a minor impurity.

**Isolation of TNF Inducing Tryptic Peptide**—We have demonstrated previously that digestion of Vmp A1 with proteinase K had no effect on the TNF inducing activity (12). We also discovered that in the process of generating tryptic peptides for NH2-terminal sequencing the TNF inducing peptide was lost after RP-HPLC, despite the digest retaining TNF inducing activity. Since a lipid is essential for activity we reasoned that the active peptide should carry the lipid modification and hence would be extremely hydrophobic. Using a palmitic acid labeled preparation of purified Vmp A1 we established a methodology to isolate the active tryptic peptide (Fig. 2A). After trypsin treatment the radioactivity and TNF inducing activity could be partitioned into chloroform:methanol, 2:1, and fractionated by normal phase HPLC using hexane, 2-propanol, 0.1% trifluoroacetic acid in water gradient. There was a single peak of TNF inducing activity eluting at 45 to 47 min that was coincident with the principal peak of radioactivity. Thirty-five percent of total activity was recovered in fractions 44 to 48. This result conclusively links the TNF inducing activity of the Vmp A1 lipoprotein with its lipid moiety. The absorbance profile at 214 nm for this peak was biphasic in nature indicating that it contained at least two components. For this reason fractions 44 to 48 from an unlabeled preparation were screened by MALDI-TOF. The spectra of the most active fractions 45 and 46 (Fig. 2B) showed a group of signals in the 1500 dalton mass range that were absent from inactive flanking fractions. Interpretation of these spectra revealed that both these TNF inducing
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sequence this peptide occurs at the amino terminus of the protein, and the deduced amino acid on the NH$_2$-terminal side of the asparagine is cysteine. Thus the 893 signal observed in Fig. 3B corresponds in mass to a N-palmitoyl S-dipalmitoyl glyceryl cysteine residue. All of the COOH-terminal ions in Fig. 3B, as well as the NH$_2$-terminal fragment ions at m/z 893, 1007, and 1094 give proof of localization of the mass increment on the NH$_2$-terminal cysteine residue. One palmitoyl residue is on the amino-terminal group itself since a doubly charged ion, rather than the singly charged 1524, would have been observed in this analysis if the NH$_2$ terminus had been free.

MS/MS analysis of the m/z 1550 group of signals in Fig. 3A showed identical COOH-terminal (y$^+$) fragment ions, together with signals equivalent to the m/z 893 ion at m/z 919 and 921 (data not shown). These data are entirely consistent with the following lipid modifications for these peptides C16:0, C16:0, C18:1 glyceryl cysteine, and C16:0, C16:0, C18:0 glyceryl cysteine. Similar results were obtained in the MS/MS analysis of fraction 46.

**DISCUSSION**

The discovery that the lipid moiety is essential for the potent cytokine inducing and mitogenic properties of bacterial lipoproteins has renewed interest in their structure. Since the seminal paper of Hantke and Braun (25) describing the structure of the lipid modification of *E. coli* murein lipoprotein several other lipoproteins have been characterized (26–28). These recent investigations have revealed significant differences in the structure of the lipid modification of bacterial lipoproteins. Moreover, the biological potency of bacterial lipoproteins varies considerably and this variation results from the type of lipid modification present on the lipoprotein (28, 29). It has been widely assumed that spirochetal lipoproteins possess a lipid modification similar to *E. coli* murein lipoprotein having three palmitic acid residues (Pam3Cys). Given the importance ascribed to spirochetal lipoproteins in the pathogenesis of disease we undertook a detailed structural characterization of a native spirochetal lipoprotein. Our results clearly demonstrate that the TNF inducing activity of *B. recurrentis* Vmp A1 resides in the NH$_2$-terminal lipid modification that is indeed similar in structure to that of *E. coli* murein lipoprotein.

Analysis by mass spectrometry revealed that Vmp A1 is not a single entity, but consists of three lipoproteins having identical peptide sequence but differing lipid moieties. The first hint of heterogeneity in Vmp A1 came from the Q-TOF analysis of the intact protein when molecules of masses 35,077, 35,101, and 35,120 daltons were observed. These molecules are of greater molecular weight than predicted from the gene sequence, confirming the presence of one or more post-translational modifications on Vmp A1. To elucidate the structure of the post-translational lipid modification of Vmp A1 that confers bioactivity, a methodology to isolate the TNF inducing active lipoprotein was established utilizing a normal phase HPLC system. Three lipoproteins of masses 1524, 1550, and 1552 daltons were observed in the active fractions. Characterization of these lipoproteins by MS/MS showed unequivocally that the lipid moieties are located on the NH$_2$-terminal peptide. Comparison of the actual mass data on the isolated lipopptide with the gene sequence revealed the presence of three types of lipid attachment of masses 789, 815, and 817 daltons. These masses are consistent with the following fatty acid substitutions to a NH$_2$-terminal glyceryl cysteine: 1) C16:0, C16:0, C16:0; 2) C16:0, C16:0, C18:1 glyceryl cysteine, and 3) C16:0, C16:0, C18:0 glyceryl cysteine. Similar results were obtained in the MS/MS analysis of fraction 46.

**Characterization of TNF Inducing Lipoprotein by Mass Spectrometry**—To determine the structure of the TNF inducing molecules of masses 1524, 1550, and 1552 daltons tandem mass spectrometry was performed on the Q-TOF instrument. First, it was necessary to discover whether the anticipated lipopeptide would ionize by electrospray, and the nanospray ES-MS spectrum of fraction 45 is shown in Fig. 3A. Because of the acidic (0.1% trifluoroacetic acid) solvent used, sodium adduct formation is minimized, and as expected two groups of signals are observed at m/z 1524 and 1550. The group of signals at 1550 includes a weak 1552 signal. Collisionally activated decomposition spectra were then derived in the MS/MS mode for the signal at m/z 1524, producing the MS/MS spectrum shown in Fig. 3B. This spectrum is interpreted as follows: clear COOH-terminal (y$^+$) ions are observed for the peptide fragmentation at m/z 632, 518, 431, 374, 317, 260, and 147 identifying a sequence, XNSGGGKIK for the tryptic peptide, where X has an NH$_2$-terminal (b-ion) mass of 893 daltons. From the vmp A1 fractions contained three molecules that ionized to give signals at m/z 1524, 1550, and a weaker 1552. The other signals present in these spectra correspond to the +1 and +2 sodium adducts of these molecules. Interestingly, the relative abundance of the three molecules differs in these fractions.

**Fig. 2. Isolation of TNF inducing tryptic peptide by normal phase HPLC.** A, fractions from the normal phase HPLC separation of a tryptic digest of palmitic acid labeled Vmp A1 were assayed for TNF inducing activity on MM6 cells at a dilution of 1/5000. The coincidence of peaks of radioactivity (dpm) and UV absorbance (A214) with bioactivity confirm that the TNF inducing component of trypsin-digested Vmp A1 is a lipopeptide. B, analysis of Fractions 45 (left panel) and 46 (right panel) from the normal phase HPLC separation of a tryptic digest by matrix-assisted laser desorption ionization mass spectrometry. Interpretation of these spectra reveals three TNF inducing lipopeptides in these fractions of m/z 1524, 1550, and 1552. Other signals correspond to the +1 and +2 sodium adducts of these ions. Numbers 1–8 indicate ions having m/z of 1524, 1546, 1550, 1552, 1568, 1572, 1574, and 1594.
was detected in the fatty acid analysis of lipoprotein extracts from other spirochetes but was not found in *E. coli* murein lipoprotein (25, 32). In conclusion, analysis of the intact lipoprotein and active lipopeptide has revealed that Vmp A1 has only one post-translational lipid modification occurring at the NH₂ terminus that confers bioactivity. Moreover our data provide the first direct proof of localization of the triacyl moiety at the NH₂-terminal cysteine residue of a lipoprotein, with one of the fatty acyl groups being on the amino-terminal group itself.

Clinical investigations of the Jarisch-Herxheimer reaction of louse borne relapsing fever have provided perhaps the clearest example to date of the causal role of TNF in the pathogenesis of severe infectious disease in humans (13, 33). We have extended this investigation by providing a comprehensive structural description of the native TNF-inducing moiety of *B. recurrentis* isolate A1. A key issue raised by this structural study is the mode of presentation of the TNF-inducing lipid component of Vmp A1 to the responsive host cell. Purified spirochetal lipoproteins and synthetic lipopeptides stimulate cells through toll-like receptor 2 (34, 35) and signaling is facilitated by CD14 (36–38). However, when Vmps are released upon lysis of the spirochetes they are presumably still associated with other membrane components, possibly forming micelles or larger aggregates. Thus the active lipid component may remain embedded within a hydrophobic structure and apparently unable to interact directly with protein receptors on the responsive cell surface. Because of the importance of *B. recurrentis* Vmp and other spirochetal lipoproteins in disease pathogenesis a fuller understanding of how this class of lipid toxin interacts with host cells is required.

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