Reports


14. Templates for in vitro synthesis of primary transcripts of tRNA\(^{\text{Met}}\) were made from ptx62 DNA (13) by polymerase chain reaction (PCR) with primers 5'-GGGAAATTGTCATATTAGCAGATACGAGCCCGCTCAATGGCAG-3' and 5'-GGGAAATTCTGCTTGCCGAGCTCCTGGCGGACAGA-3'.

15. Precursors with an intron or extra sequences at the 5' end are processed with 3' CCA end (23).

16. If tRNAs remain aminoacylated during translocation through the nuclear pore complex, they could be exported slowly [M. Zasloff, Proc. Natl. Acad. Sci. U.S.A. 80, 6439 (1983); J. A. Tobian, L. Drinkard, M. Zasloff, Cell 43, 415 (1985)]. C. Traboni, G. Ciliberto, R. Cortese, ibid. 36, 179 (1984); [23]. Mutations at position 55 were not tested previously, but they alter the 5'-box of the tRNA promoter, which is required for producing tRNAs in vivo from injected genes.

17. For blockage of aminoacylation or protein synthesis, aminoacyl-AMS compounds. Supported by NIH grants GM30200.


Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene

Alexander Poltorak, Xiaolong He,† Irina Smirnova, Mu-Ya Liu,‡ Christophe Van Huelfe,† Xin Du, Dale Birdwell, Erica Alejos, Maria Silva, Chris Galanos, Marina Freudenberg, Paola Ricciardi-Castagnoli, Betsy Layton, Bruce Beutler

Mutations of the gene Lps\(^{\text{C3H}}\) specifically impede lipopolysaccharide (LPS) signal transduction in C3H/HeJ and C57BL/10ScCr mice, rendering them resistant to endotoxin yet highly susceptible to Gram-negative infection. The codominant Lps\(^{\text{C3H}}\) allele of C3H/HeJ mice was shown to correspond to a missense mutation in the third exon of the Toll-like receptor-4 gene (Tlr4), predicted to replace a charged arginine (Arg) with a leucine (Leu) (717). In Xenopus oocytes, a larger nuclear volume may make this less of a problem.


21. If tRNAs remain aminoacylated during translocation through the nuclear pore complex, they could be exported slowly [M. Zasloff, Proc. Natl. Acad. Sci. U.S.A. 80, 6439 (1983); J. A. Tobian, L. Drinkard, M. Zasloff, Cell 43, 415 (1985)]. C. Traboni, G. Ciliberto, R. Cortese, ibid. 36, 179 (1984); [23]. Mutations at position 55 were not tested previously, but they alter the 5'-box of the tRNA promoter, which is required for producing tRNAs in vivo from injected genes.


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sepsis. Nonetheless, it is clear that timely recognition of LPS by cells of the innate immune system permits effective clearance of a Gram-negative infection before it becomes widely disseminated (2, 3).

More than 30 years ago, mice of the C3H/HeJ strain were found to have a defective response to bacterial endotoxin (4–6). Inquiry into the genetic basis of LPS resistance revealed a single locus (Lps), wherein homozygosity for a codominant allele (Lps<sup>s</sup>) was responsible for the endotoxin-unresponsive state. The Lps<sup>s</sup> mutation arose in mice of the C3H/HeJ substrain and became fixed in the population during the early 1960s (9). In contrast to C3H/HeJ mice, substrains C3H/HeN and C3H/OUJ (Lps<sup>x</sup> homozygotes), which diverged from the same stock as C3H/HeJ mice, exhibit vigorous responses to LPS.

A second mutation preventing responses to endotoxin was identified in mice of the strain C57BL/10ScCr (10–12); animals of the control strain C57BL/10ScSn are normally responsive. The allelic nature of the C3H/HeJ and C57BL/10ScSn mutations was indicated by the observation that F<sub>1</sub> animals produced by the cross C57BL/10ScCr × C3H/HeJ are as unresponsive as individuals of the C3H/HeJ parental strain (10). But significantly, heterozygotes produced by the cross C57BL/10ScCr × C57BL/10ScSn are as responsive to LPS as the normal (C57BL/10ScSn) parent (10), indicating that the C57BL/10ScCr allele is not codominant, but is strictly recessive to the common wild-type allele.

Speculations regarding the protein that is affected by mutations of Lps have, for the most part, posited that the LPS signal transduction apparatus is disrupted. Ulevitch, Tobias, Wright, and co-workers showed that LPS is concentrated from the plasma by lipopolysaccharide binding protein (LBP), and that the genetically unlinked plasma membrane protein protein CD14 is the principal receptor for LPS on the surface of mononuclear cells (13–15). Deletion of the CD14 gene substantially increases the concentration of LPS required for a biological response (16). However, because CD14 lacks a cytoplasmic domain, it has been postulated that a coreceptor for LPS must permit transduction of the signal across the plasma membrane. Several protein kinase cascades are known to become activated by LPS (17–21), ultimately leading to the production of TNF and other cytokines that mediate LPS effects (22, 23). However, direct biochemical, immunologic, and expression cloning approaches have failed to identify the genetic lesions in endotoxin-resistant mice.

In 1978, the Lps locus was mapped to mouse chromosome 4 and shown to occupy a position between the Mip-1 and Ps loci (24, 25). Our own genetic and physical mapping data (26) identified two limiting genetic markers (B and 83.3) that were separated from Lps<sup>s</sup> by, respectively, four crossovers in a panel of 1600 meioses and three crossovers in a panel of 493 meioses.

A minimal contig, consisting of 20 bacterial artificial chromosome (BAC) clones and one yeast artificial chromosome (YAC) clone, was analyzed by sequencing. Nearly 40,000 reads were obtained from shotgun-cloned genomic DNA, bringing over 1.6 Mb of the central contig to a near-contiguous state and yielding dense coverage of >95% of the entire critical region. BLAST searches (27) performed on masked versions of the sequence disclosed dozens of high-scoring homologies with published expressed sequence tags (ESTs), but these were excluded from consideration as they could not be cloned from macrophage or fetal CDNA libraries of reliable complexity. Several pseudogenes were observed, but were dismissed because they were found to be fragmentary. GRAIL analyses, performed on long, contiguous sequences of the central contig with the program X-GRAIL (28), revealed an abundance of retroviral repeats and scattered non-retroviral exons, many of which proved to be derived from pseudogenes.

Only two authentic genes (a portion of the Pappa locus and the complete Tlr4 locus) were identified in the entire region, each by BLAST analysis and by GRAIL analysis (Fig. 1). Pappa encodes a secreted metalloproteinase and is not expressed by primary macrophages or macrophage cell lines (29). These considerations, as well as its extreme proximity to marker B, made it seem a poor candidate. Tlr4 seemed an excellent candidate, both on the grounds of map position and because the proinflammatory interleukin-1 (IL-1) receptor, like Tlr4, is a member of the Toll receptor family. Further, a human mutation causing coresistance to LPS and IL-1 (30) attests to the likelihood that the IL-1 and LPS use structurally related receptors.

Accordingly, we cloned the Tlr4 cDNAs from C3H/HeJ mRNA and from the mRNA of several LPS-responsive strains of mice (including mouse/rat 712

<table>
<thead>
<tr>
<th>mouse/rat</th>
<th>C2HHeJ(700)</th>
<th>RFHLCLHRYDFI</th>
<th>H</th>
<th>GVAIAAIQIQEGFHKS... (730)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeN(700)</td>
<td>RFHLCLHRYDFI</td>
<td>P</td>
<td>GVAIAAIQIQEGFHKS... (730)</td>
<td></td>
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<tr>
<td>rat (700)</td>
<td>RFQLCLHRYDFI</td>
<td>P</td>
<td>GVAIAAIQIQEGFHKS... (730)</td>
<td></td>
</tr>
<tr>
<td>human (702)</td>
<td>PFQLCLHRYDFI</td>
<td>P</td>
<td>GVAIAAIQIQEGFHKS... (732)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Diagram of a small portion of the 2.6-Mb contig spanning the Lps critical region, described elsewhere in its entirety along with the sequences of primer pairs used to amplify markers (26). Centromere is to the left. Three BACs (49K20, 309I17, and 152C16; Research Genetics designations) contain fragments of Pappa (49K20) and the complete Tlr4 gene (309I17 and 152C16). Each BAC is ~150 kb long. The orientation and exonic composition of the genes identified is schematically correct, but for clarity, the genes are drawn at far higher magnification than the BACs. Dots to the left of the three Pappa exons that were identified in K20 indicate that the gene continues to the left of the contig [other exons were detected in BACs 133I6, 216C14, and 35B4 (29)]. Genetic mapping data (number of crossovers per number of meioses examined) are bracketed above the two genetic markers nearest to the Lps<sup>s</sup> (B and D4MIT178).

Fig. 2. The Lps<sup>s</sup> allele represents a missense mutation affecting the cysteoplastic domain of Tlr4. Reverse transcription of mRNA isolated from mouse peritoneal macrophages was performed by PCR with the primers TCTAACCCTCTCCCTGCGAC and CCTCTTCCTCTCCATGAAAG, which amplify the entire coding region of the mouse Tlr4 gene, yielding a product 2951 nucleotides (nt) long. The open reading frame of the mouse Tlr4 cDNA predicts a protein that is 835 amino acids long. The mutation, at position 712, lies in the most conserved portion of the Tlr4 sequence and is located within the cysteoplastic domain. Dots below the sequence indicate residues that vary between species. The Pro→His substitution that distinguishes Tlr4 of C3H/HeJ mice is boxed. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
ing C3H/HeN) by reverse transcriptase–polymerase chain reaction, using primers derived from the genomic sequence. A single mutation (the presence of an A instead of a C) was observed at position 2342 of the C3H/HeJ Tlr4 cDNA sequence (GenBank accession number AF095353). This mutation lies within the coding region: At position 712 (within the cytoplasmic domain), a histidine is predicted to occur in the Tlr4 protein of C3H/HeJ mice, whereas LPS-responsive mice, rats, and humans display a proline in this position (Fig. 2) (31). The same mutation was identified in C3H/HeJ genomic DNA, but not in genomic DNA from C3H/HeN mice or mice of any other strain examined (29).

Although the Tlr4 cDNA was readily amplified by RT-PCR from macrophage RNA derived from C3H/HeJ, C3H/HeN, and C57BL/10ScSn mice, it could not be amplified from macrophage RNA derived from C57BL/10ScCr mice. In contrast, a low-abundance control cDNA (32) could be readily amplified from all strains (Fig. 3A). Moreover, the Tlr4 mRNA could be detected on Northern (RNA) blots prepared with total RNA derived from macrophages of C57BL/10ScSn mice but not C57BL/10ScCr mice (Fig. 3B).

Because a definable mutation exists within Tlr4 in C3H/HeJ mice, and complete absence of Tlr4 mRNA expression is observed in C57BL/10ScCr mice, it is apparent that Lps is identical to Tlr4. Certain inferences may thus be drawn from the phenotypes that result from distinct allelic combinations of Lps.

The null allele of Lps represented in C57BL/10ScCr mice behaves as a recessive mutation (10). Hence, the presence of a single wild-type Tlr4 allele (Tlr4<sup>+/+</sup>) is sufficient to permit normal LPS signal transduction. By contrast, the mutation of C3H/HeJ mice is codominant, in the sense that Tlr4<sup>+/+</sup>/Tlr4<sup>+/−</sup> heterozygotes show intermediate levels of endotoxin response (7). Thus, the Pro→His point mutation exerts a dominant negative effect on LPS signal transduction.

A single copy of the Lps<sup>e</sup> allele (Tlr4<sup>+/−</sup>) yields a phenotype as unresponsive as two copies (Tlr4<sup>+/−</sup>/Tlr4<sup>+/−</sup>) (10). This fact is consistent with the notion that LPS signal transduction proceeds directly through the Tlr4 molecule, and tends to detract from the alternative hypothesis that Tlr4 undergoes interaction with a second plasma membrane protein that acts, in turn, as an LPS signal transducer.

Tlr4 mRNA is reportedly expressed predominantly in lymphoid tissues (33). Our own data (29) are in agreement with this finding, but in addition (Fig. 4) suggest that the Tlr4 mRNA is strongly and transiently suppressed by LPS in RAW 264.7 cells (34). As such, down-regulation of Tlr4 mRNA may contribute to endotoxin tolerance (35). It remains to be seen whether species-dependent variation in LPS responses, and modulation of LPS sensitivity by steroids, interferon-γ, and other agents, may be traced to the Tlr4 protein.

LPS signal transduction via Tlr4 has not previously been observed in any experimental system. However, it has recently been reported that human Tlr2 cDNA transfected into 293 cells can promote LPS signal transduction, given coexpression of CD14 (36). The present study excludes an independent role for Tlr2 in LPS signal transduction. The demonstration that Lps is identical to Tlr4 effectively proves that Tlr4 is essential for LPS signaling. And in mice that lack Tlr4 (for example, C57BL/10ScCr animals), endogenously expressed Tlr2 does not contribute appreciably to LPS signal transduction, which fails to occur at measurable levels despite the presumption that the Tlr2 locus is intact. Although Tlr2, like Tlr4, might be required for LPS signaling, the available data are not sufficient to sustain this conclusion.

In Drosophila, the Toll signaling pathway culminates in activation of the drosomycin gene and is required for effective protection against fungal infection (37). Several homologs of the prototypic gene Toll exist in Drosophila, including 18-wheeler (38, 39), which facilitates the antibacterial response of flies (40). In mice, Tlr4 appears to have been retained chiefly to serve the LPS response pathway. Hence, C3H/HeJ and C57BL/10ScCr mice are developmentally and immunologically normal, aside from their inability to respond to LPS and to counter Gram-negative infection. CD14, the best-characterized cell surface receptor for LPS, is also a member of the Toll superfamily. It is conceivably that it directly engages Tlr4 upon interaction with LPS, thereby inducing signal transduction through the latter protein.

Hu and co-workers (41) have recently ad-
duced evidence to suggest that, in birds, distinct allelic forms of Lps influence survival during Gram-negative infection. It is possible that mutations of human Tlr4 also affect susceptibility to Gram-negative infection, or its clinical outcome.

References and Notes
18. J. Han, J.-D. Lee, I. Ribbs, R. J. Ulevitch, Science 265, 808 (1994).
27. BLAST searches were performed against the nonredundant (NR) GenBank database, the TIGR database of ESTs, and the dbEST database of ESTs. Searches against NR and TIGR databases were performed at both the nucleotide (blastn) and amino acid (blastx) levels. blastn searches were carried out at the nucleotide level only.
29. A. Pollorak et al., not data shown.
31. Supplementary Web material for Fig. 2 is available at www.sciencemag.org/feature/data/985613.shl.
32. To monitor the efficiency of reverse transcription and PCR, we used primers specific for the transferrin receptor (TFR) as a positive control when attempting to detect the low-abundance Tlr4 mRNA in macrophage or fetal RNA samples by RT-PCR.
34. RAW264.7 cells, obtained from the American Type Culture Collection, are immortalized LPS-responsive cells, frequently used in studies of LPS signal transduction and TNF gene regulation. RAW 264.7 cells, like primary macrophages, become refractory to LPS for a variable interval of time after a primary stimulus with LPS.
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42. We acknowledge the assistance of J. Turner, A. Powelka, R. Jain, R. Clisch, and C. Brady, all summer undergraduates who worked with us to identify the lps mutation. We are also grateful to the Beutler Family Trust for providing funds for the purchase of an ABI model 373 sequencer.

Exploiting the Basis of Proline Recognition by SH3 and WW Domains: Design of N-Substituted Inhibitors
Jack T. Nguyen, Christoph W. Turck, Fred E. Cohen, Ronald N. Zuckermann, Wendell A. Lim*

Srp homology 3 (SH3) and WW protein interaction domains bind specific proline-rich sequences. However, instead of recognizing critical prolines on the basis of side chain shape or rigidity, these domains broadly acceptedamide N-substituted residues. Proline is apparently specifically selected in vivo, despite low complementarity, because it is the only endogenous N-substituted amino acid. This discriminatory mechanism explains how these domains achieve specific but low-affinity recognition, a property that is necessary for transient signaling interactions. The mechanism can be exploited: screening a series of ligands in which key prolines were replaced by unnatural N-substituted residues yielded a ligand that selectively bound the Grb2 SH3 domain with 100 times greater affinity.

Table 1. Reduction in binding affinity (27) caused by alanine (A) or sarcosine (A*) substitutions within in proline-rich ligands of Sem5 SH3 domain and Yap WW domain (22). “Required” prolines are underlined.
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