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The Miller Hypothesis

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Abstract

The immune response is a cornerstone in the body's struggle against microbial pathogens. In ways that we do not yet completely understand, the mammalian immune response has evolved to identify proteins of pathogens that are either important virulence factors or key immunoprotective targets. Professor James N. Miller suggested that one way to discover such proteins is to harness the power of the immune system in the laboratory. This general concept, referred to here as the Miller Hypothesis, took several different manifestations in the discovery of some of the best known and widely studied leptospiral proteins: The porin OmpL1 was identified by surface immunoprecipitation, leptospiral immunoglobulin-like proteins were uncovered by screening a genomic library with sera from leptospirosis patients, and the major outer-membrane lipoprotein LipL32 was recognized through immunoblot studies. Such approaches will continue to bear fruit for both the leptospiral research field and research on other invasive pathogens.

Keywords

immunoprotection; *Leptospira interrogans*; surface immunoprecipitation; surfaceome; surface protein; transposon sequencing; *Treponema pallidum*

I. INTRODUCTION

My nearly 30-year career in research on spirochetes was launched during my training with Professor James N. Miller at the University of California at Los Angeles from 1987 to 1991. The themes of my research, indeed many of my best insights, trace their origin to conversations with Prof. Miller. One theme that dominated many of our conversations was how understanding and characterizing the humoral immune response could help to identify spirochetal surface proteins. Prof. Miller's laboratory was a World Health Organization reference laboratory for a serological assay that was considered to be the gold standard for the diagnosis of syphilis: the *Treponema pallidum* immobilization (TPI) test. In the TPI test, serum from a patient suspected of syphilis is incubated with *T. pallidum* and guinea pig serum as a source of complement. After 16 hr of incubation, the test was read by assessing *T. pallidum* motility by dark-field microscopy. Patients with a history of syphilis inevitably form treponemicidal antibodies directed toward *T. pallidum* surface proteins. The remarkable ability of the immune system to target key microbial antigens led to discussions on how to harness the antibody response to discover spirochetal surface proteins that were significant, for either understanding pathogenesis or developing vaccines. I refer to this

general approach as the Miller Hypothesis. Here, I describe three of the most interesting and important leptospiral outer-membrane proteins discovered by applying this approach.

II. LEPTOSPIRAL SURFACE PROTEINS

A major focus of our work has been to identify and characterize leptospiral surface proteins. Surface proteins are a double-edged sword for pathogens, including invasive spirochetes that persist in the mammalian host for long periods of time. On one hand, surface proteins serve to facilitate interactions with the host that benefit the pathogen. On the other hand, surface proteins serve as targets for a protective immune response. When we began our work in the late 1980s, leptospiral lipopolysaccharide (LPS) was understood to be present on the leptospiral surface, but little was known about leptospiral surface proteins.

Our initial approach to using the power of the Miller Hypothesis to discover surface proteins involved a technique called surface immunoprecipitation. First, we hyperimmunized rabbits with whole leptospires to generate antibodies to all possible leptospiral proteins. When these antibodies were allowed to bind to intact leptospires, they quickly caused the bacteria to clump and precipitate by cross-linking LPS from different organisms. These precipitates were gently washed to remove unbound antibodies and avoid disruption of the bacteria, which might expose subsurface antigens. Antibody–antigen complexes were harvested by dissolving the outer membrane with Triton X-100 and allowing the antibodies to bind to protein A sepharose. The antibody–antigen complexes were analyzed by immunoblots, revealing LPS and three distinct proteins that eventually became known as OmpL1, LipL41, and LipL46.¹

III. THE LEPTOSPIRAL PORIN OmpL1

From the beginning, OmpL1 seemed like an intriguing surface protein for further study. Although OmpL1 is one of the most abundant leptospiral membrane proteins,² twice as much OmpL1 was recovered by surface immunoprecipitation from attenuated organisms than from virulent organisms.³ This difference correlated with the number of intra-membranous particles observed by freeze-fracture electron microscopy, suggesting that OmpL1 could be a porin. This suspicion was supported by the OmpL1 sequence, which contained ten OMP-like transmembrane sequences, and five intervening surface-exposed loops. Eventually, we obtained direct evidence of porin activity by measuring changes in electrical conductivity during addition of recombinant OmpL1 to lipid bilayers.⁴ Many *ompL1* genes were subsequently found to be mosaics, resulting from relatively frequent horizontal gene transfer events among leptospiral species, a mechanism similar to that described for the PorB porins of the *Neisseria* species.⁵

What could have selected for these interspecies gene transfer events? Immunological pressure is the most likely explanation. The OmpL1 region that was frequently involved in horizontal gene transfer and exhibited the highest degree of sequence variability was the first and largest of the OmpL1 surface-exposed loops (Fig. 1). This suggests that varying the OmpL1 sequence provides some selective advantage, perhaps by evading the host immune response to the first surface-exposed loop. In addition, vaccine studies support the

conclusion that when presented in the proper configuration, OmpL1 provides immunoprotection against leptospirosis.⁶⁷ In addition to its porin function, OmpL1 may also have a role in interactions with host cells and tissues. In a comparison with four other leptospiral proteins, only OmpL1 bound significantly to mammalian cells.⁸ The mammalian cell components for which OmpL1 had special predilection turned out to be glycosaminoglycans (GAGs), especially heparin and heparan sulfate, which are GAGs with high negative charge densities.

IV. LEPTOSPIRAL IMMUNOGLOBULIN-LIKE PROTEINS

We sought to discover proteins whose expression is up-regulated or expressed exclusively during infection and poorly expressed or not expressed at all when organisms are grown in culture. To achieve this goal, we applied the Miller Hypothesis by using sera from patients with leptospirosis to screen a genomic expression library for antigens recognized during infection. This approach resulted in the identification of a family of proteins comprised of a series of immunoglobulin-like domains.⁹ Consistent with their identification by antibodies formed during leptospirosis, these leptospiral immunoglobulin-like (Lig) proteins were found to be excellent serodiagnostic antigens¹⁰ and represent some of the most prominent protein antigens recognized early in the immune response to leptospirosis.¹¹ LigA is composed of a series of 13 immunoglobulin-like domains, whereas the highly related LigB protein has 12 immunoglobulinlike domains followed by a large carboxy-terminal region. Although the immunoglobulin-like domains of *ligA* and *ligB* are highly related, they exhibit significant amino acid sequence differences.¹² For example, only the terminal immunoglobulin-like domains of *ligA* are highly protective from lethal infection in the hamster model of leptospirosis.¹³ Of these terminal domains, *ligA* domains 10–12 are both required and sufficient for immunoprotection.¹⁴

Consistent with a role of LigA and LigB during infection was the discovery that their expression responds dramatically to osmolarity and temperature. Leptospiral medium has a relatively low osmolarity of 70 mOsm, whereas the osmolarity of serum is ~300 mOsm. When the osmolarity of the medium is shifted to physiologic levels by addition of salt or sucrose, there is a dramatic increase in *lig* gene transcription. At ambient temperature, *lig* transcripts are poorly translated because of the secondary structure, in which a messenger RNA (mRNA) hairpin loop obscures the ribosome-binding site and start codon (Fig. 2A). We confirmed the RNA thermometer hypothesis in *Escherichia coli* using constructs fusing the *ligA* untranslated region (UTR) with a β -galactosidase reporter. The mRNA leader and first six codons of the *ligA* gene in frame with *IctcZ* were cloned into the pBAD vector under control of the arabinose promoter. We observed a strong 13-fold increase in β -gal activity at 37°C compared to that at 30°C with the *lig* construct. This compared well to the 14- to 20-fold increase in Lig protein expression after shifting leptospiral cultures from 30°C to 37°C (Fig. 2B). The thermoregulatory region was localized using a deletion construct lacking all of the *lig* UTR except for the hairpin loop. The dramatic responses of LigB expression to osmolarity and temperature suggest that it may be involved in the transition from outside to inside the host.

One of the first indications of the kind of functions in which Lig proteins were involved was that leptospiral adhesion to extracellular matrix proteins and fibrinogen was induced by increasing osmolarity to physiologic levels.¹⁵ Subsequent studies showed that much of this increased adhesion was probably due to increases in Lig protein. Transformation of the nonpathogenic saprophyte *Leptospira biflexa* with either the *ligA* or *ligB* gene significantly increased adhesion to fibrinogen.¹⁶ In addition, purified recombinant LigB fragments were found to bind to a variety of proteins including fibrinogen, fibronectin, and collagen, and the region of LigB involved in binding was localized to domains 9–11. It was surprising as to the amount of structurally different proteins to which LigB was able to bind with relatively high avidity. This suggested that LigB's binding activity violates the standard lock-and-key model of protein-protein interactions. The lock-and-key model presumes that protein shapes are relatively static and inflexible. In contrast, fibronectin-binding proteins from *Streptococcus pyogenes* and *Staphylococcus aureus* use a flexible tandem β zipper to adapt to the shape of fibronectin.¹⁷ LigB's relatively promiscuous interactions may have a similar explanation based on the β -sandwich structure of its immunoglobulin-like domains and interdomain angle flexibility.¹⁸

The *ligA* and *ligB* genes are highly related and appear to have resulted from a gene duplication event.¹² The *ligB* gene is found in all pathogenic leptospires, whereas the *ligA* gene has been lost from certain leptospiral lineages. Although this may suggest that the *ligB* gene is required for infection, mutants lacking *ligB* retain virulence in animal models of infection.¹⁹ This lack of requirement for *ligB* during infection has been confirmed in a high-throughput massively parallel transposon sequencing study.²⁰ We examined the *in vivo* fitness of a pool of 42 defined mini-Himar transposon insertion mutants, including mutants with insertions in genes encoding LigB, signal transduction proteins, flagellar genes, and *loa22*, a gene previously shown to be required for virulence.²¹ The 42 mutants were grown individually in Ellinghausen–McCullough–Johnson–Harris and counted and pooled together in equal amounts before being inoculated intraperitoneally into eight hamsters (input pool = 10^6 total leptospires/animal). Four days postchallenge, DNA from blood, kidney, and liver (output pools) was extracted and processed for Illumina sequencing. Sequences were filtered for quality, mapped, and enumerated as a percentage of total sequences in the input pool and output pools. Competitive indices (output pool %/input pool %) across mutants were normalized by setting the median ratio to 1.0 for the 42 mutants in each animal. The *loa22* (*lic10191*) mutant exhibited decreased fitness ($p < 0.01$) along with mutants with transposon insertions in three regulatory genes (*lic12031*, *lic12324*, and *lic12327*) and several intergenic locations. However, as predicted by previous studies, the *ligB* mutant exhibited normal *in vivo* fitness.

Given the dramatic induction of LigB expression in response to host-like environmental conditions (osmolarity and temperature), the strong early antibody response to LigB, and its unique functional characteristics, it came as a surprise that LigB was not required for infection. An explanation for this conundrum came from a study involving development of an innovative tool for manipulating gene expression: transcription activator-like effectors (TALEs).²² TALEs can be engineered to bind practically any desired DNA sequence. When TALE genes were constructed with specificity for the homologous regions of both the *ligA* and *ligB* promoters and expressed constitutively in *Leptospira interrogans* serovar Manilae, 2-

to 9-fold reductions in expression of LigA and LigB were observed. *L. interrogans* clones expressing the lig promoter-specific TALEs were found to be avirulent and unable to infect animal tissues, including the kidney. This result suggests that LigA and LigB have redundant functions, which is not surprising given their sequence similarities, and explains the previous studies showing that *ligB* mutants retain virulence.

V. THE MAJOR OMP LipL32

LipL32 was discovered as a result of yet another approach to application of the Miller Hypothesis: A serosurvey of leptospiral immunoblot antigens recognized by the humoral immune response of patients with leptospirosis.²³ When the total protein profile of whole leptospiral cells was separated by sodium dodecyl sulfate–polyacrylamide electrophoresis, the 32-kDa band corresponding to LipL32 stood out above all others, in terms of both sensitivity and specificity. Cloning and sequencing of the *lipL32* gene revealed a fatty acid–modified lipoprotein found exclusively in the leptospiral outer membrane based on cell fractionation using either the detergent Triton X-114²⁴ or sucrose gradient ultracentrifugation.²⁵ Part of the reason for its immunodominance is that LipL32 is the most abundant protein in *L. interrogans*: There are more than 38,000 copies/cell based on quantitative matrix-assisted laser desorption/ionization–time of flight.² LipL32 is expressed at high levels both in cultivated leptospires and during infection, making it a useful target for immunohistochemistry.²⁴ Initial studies involving surface labeling, immunoelectron microscopy, and surface enzyme-linked immunoabsorbent assays suggested that LipL32 was a component of the leptospiral surfaceome. Subsequently, carefully performed surface immunofluorescence and surface proteolysis studies determined LipL32 to be largely, if not entirely, subsurface.²⁶ Thus, the consensus now is that as a lipoprotein, LipL32 is restricted to the inner leaflet of the outer membrane. This subsurface location is difficult to reconcile with studies describing interactions of LipL32 with host proteins.^{27,28} LipL32's subsurface location is probably also the reason why most vaccine studies have failed to demonstrate immunoprotection with LipL32.²⁹

The combination of subsurface outer-membrane location, its abundance, and a high level of sequence conservation across pathogenic leptospires⁵ would suggest that LipL32 has a critical structural role, for example, in outer-membrane stability. For these reasons, it is surprising that LipL32 knockout mutants were morphologically intact, fully virulent for hamsters, and able to produce chronic renal infection in rats.³⁰ The enigma of LipL32 is that we do not understand its true function nor why pathogenic leptospires would invest so much metabolic capital in producing large amounts of a protein that is not required for infection. Crystal structure studies indicate that LipL32 is a dimer with two patches of electronegative surface charge that appear to be involved in calcium binding.³¹ One possibility is that LipL32 is important for maintaining the local calcium concentration to stabilize outer-membrane phospholipids. In his review on this subject,²⁹ Gerald Murray suggests that “LipL32 may have an essential role in aspects of virulence not tested in the current animal models.” Survival in the aquatic environment is a well-known capability of pathogenic leptospires that is key to transmission among mammalian hosts. We found that *L. interrogans* was able to survive for up to 1 yr in distilled water in the presence of 0.5% agarose.³² It would be interesting to test the survival of the LipL32 mutant in distilled water,

perhaps with a small concentration of ethylenediaminetetraacetic acid added to compete with LipL32 for calcium.

VI. CONCLUSIONS

I am grateful for the opportunity to receive my research training with Professor James N. Miller, whose insights into the humoral immune response as a tool for understanding pathogen surface proteins helped guide my career in bacterial pathogenesis. Three of the most immunodominant leptospiral proteins were discovered using antibodies generated during the immune response to either leptospiral cells or leptospirosis. The pull-down assay known as surface immunoprecipitation identified the porin OmpL1, whose sequence exhibits the effects of selective pressure of the immune system on a surface-exposed protein. Genes encoding the leptospiral immunoglobulin-like Lig proteins were selected to use antisera from patients with leptospirosis. The Lig proteins are of great interest as multifunctional adhesins that can interact with a wide variety of host proteins. The major outer-membrane protein LipL32 was identified by immunoblots studies with human leptospirosis sera. Although LipL32 is the most abundant leptospiral protein, and its structure is known in great detail, its function continues to be an enigma. Application of the Miller Hypothesis is likely to continue to be highly productive in terms of understanding the microbiology and pathogenesis of microbial pathogens.

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ABBREVIATIONS:

DNA	Deoxyribonucleic acid
GAG	glycosaminoglycan
Lig	leptospiral immunoglobulin-like
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
TALE	transcription activator-like effector
TPI	<i>Treponema pallidum</i> immobilization
UTR	untranslated region

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FIG. 1:

Model of OmpL1 topology. OmpL1 membrane topology is shown using a consensus amino acid sequence based on alignment of OmpL1 sequences from 38 strains. Colors of amino acids indicate no variability, increased variability (0%–29.9%), high variability (≥ 30%), or positive selection of amino acid variability. Variable regions were found to be located in areas predicted to be surface-exposed loops. Sequence variability information was unavailable for amino acids shown in gray. VR, Variable region.

FIG. 2:

Thermoregulation of Lig expression. Lig expression is strongly regulated by temperature through a thermally sensitive mRNA hairpin loop that obscures the ribosome-binding site and ATG start codon at ambient temperature (A). Although *ligA* and *ligB* transcripts are increased by fivefold to sixfold at 37°C relative to 30°C, this posttranslational regulatory system increases LigA and LigB protein by 15- to 20-fold in response to increased temperature.