The Panton-Valentine Leukocidin Is a Virulence Factor in a Murine Model of Necrotizing Pneumonia

To the Editor—We read with great interest the article by Villaruz et al about a point mutation in the agr locus of Staphylococcus aureus causing phenotypes that we and other investigators had attributed to Panton-Valentine leukocidin (PVL) [1]. In this article, the authors stated that a strain used in one of our studies [2] “contained an unintended mutation in agr, which dramatically changed gene expression.” The authors also stated that “the virulence phenotype described by Labandeira-Rey et al for the same strain was influenced by the agr mutation and not PVL.”

The results obtained by Villaruz et al prompted us to check the integrity of the agr locus in our own strain stocks. As shown in Figure 1A, strains RN6390, LUG855 (Pvl lysogen of RN6390), LUG776 (LUG855 Δpvl), LUG862 (plasmid pvl complementation in LUG776), and LUG1564 (empty vector in LUG776) show normal β-hemolysis when plated in blood-agar medium, whereas RN6911 (the RN6390 Δagr derivative) showed no hemolysis. As described by the authors, β-hemolysis is “a common and simple read-out for agr functionality” [1]. Therefore, contrary to the results described by Villaruz et al, the LUG855 strain used in the studies of Labandeira-Rey et al displays a normal agr phenotype. In addition, we have sequenced the agr P2-P3 region (nucleotides 1724–1458) in RN6390, LUG776, and LUG855 and found that the sequences were identical to those reported for the RN6390 parental strain, NCTC8325-4. We performed Northern blot analyses to detect the expression of RNAIII transcripts as a direct readout for agr activity (Figure 1B). As expected, RNAIII transcripts were generated at similar levels in most strains tested, including LUG855, but they were not present in the Δagr RN6911 or its Pvl lysogen, LUG856. Expression analyses of protein A by use of immunoblot assays confirmed our previously published results, in which the strain LUG855 showed an increased level of protein A production compared with its isogenic Δpvl derivative (LUG776) and its parental strain (RN6390) (Figure 1C). Overexpression of spa is also seen in the Δpvl strain complemented by a plasmid encoding PVL (LUG862), but it is not seen in the vector-only control (LUG1564).

Regarding the PVL production, Villaruz et al claim that “the expression of PVL in LUG855 was very low, most likely owing to strong agr control of lukSF-PV, which was defective in LUG855.” Using a specific enzyme-linked immunosorbent assay [3], we have measured the level of lukS-PV production in the spent medium from cultures of selected strains (Figure 1D). Our results show that LUG855 produced ∼500 ng/mL culture medium during early stationary phase, whereas the plasmid-complemented strain LUG862 produced up to 5-fold more, presumably because of the multicopy nature of the expression vector used in this strain. These levels of PVL production are consistent with a functioning agr locus. By comparison, the Δagr, Pvl lysogen strain LUG856 showed a reduced level of PVL production. Therefore, we agree with the authors’ statement that lukSF-PV may be under agr regulatory control.

Finally, we have repeated experiments to compare the ability of the strains used in our studies to cause disease in a non-lethal model of murine pneumonia. As described in our previous reports, the LUG855 strain caused more morbidity (measured as weight loss) compared with its isogenic Δpvl strain (LUG776) and its parental strain (RN6390) (Figure 1E). Tissue sections from lungs infected with LUG855 showed a strong recruitment of neutrophils, necrosis, and hemorrhage (Figure 1F). Conversely, the lungs infected with the PVL-negative strain (LUG776) showed normal lung structures, despite some leukocyte infiltration (Figure 1F).

These results are analogous to those obtained when the LACΔpvl strain (constructed by M. Otto and donated by F. DeLeo) were used as infecting agents [4]. In our mouse model, the expression of PVL enhances the virulence of S. aureus, regardless of whether the infecting agents are laboratory strains or clinical isolates. Furthermore, anti-PVL antibodies effectively block the cytolytic action of PVL [5], and animals actively or passively immunized with the PVL subunits are effectively protected against community-associated methicillin-resistant S. aureus pneumonia and skin infections, which confirms the role played by PVL in such infections [4, 6].

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References

1. Villaruz AE, Bubeck Wardenburg J, Khan BA, et al. A point mutation in the agr locus rather than expression of the Panton-Valentine leukocidin caused previously reported phenotypes...
Figure 1. Characterization of *Staphylococcus aureus* strains. A, Hemolysis on sheep blood–agar plates of streaked cultures. The LUG855 plate is shown in its entirety to demonstrate a homogeneous phenotype. B, RNA detection by use of Northern blot analyses. Total RNA was electrophoresed in a 1% agarose gel containing 2.2 mol/L formaldehyde and vacuum transferred onto a nylon membrane. RNAIII and 5S ribosomal RNA were detected using specific digoxigenin-labeled RNA probes as described elsewhere [7]. C, Detection of *spa* in culture supernatants. Strains were grown in casein-casein-yeast medium at 37°C with vigorous shaking for 6 h. Production of *spa* was determined by means of Western blot analysis in standardized samples (optical density at 600 nm, 1). Proteins present in 30 μL of spent culture medium were separated on sodium dodecyl sulfate 12% polyacrylamide gels, blotted to nitrocellulose, and probed with a 1:400 dilution of mouse anti-*spa* monoclonal antibodies (Sigma-Aldrich). Bound antibody was detected with a 1:4000 dilution of goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Sigma) and followed by Western blotting detection (ECL Western blotting systems; GE). D, Panton-Valentine leukocidin (PVL) production in selected strains. The *lukS-PV* was quantified in aliquots of standardized supernatants (optical density at 600 nm, 1) by use of a solid-phase sandwich enzyme-linked immunosorbent assay, with a mouse monoclonal antibody and a rabbit peroxidase-conjugated polyclonal F(ab)2 fragment targeting *lukS-PV*, as recommended by the supplier (R&D Immunoassays, bioMerieux) [3]. E, Expression of PVL enhancing the virulence of isogenic *S. aureus* strains. Six-week-old Balb/c female mice (n = 15) were intranasally inoculated with suspension of RN6390, LUG776, or LUG855 as described elsewhere [2]. Line graphs indicate weight loss as a measure of morbidity. *P< .05 on day 2 for LUG776 vs LUG855. F, Lung tissue sections stained with hematoxilyn-eosin from representative animals infected with LUG776 and LUG855.
The expression of the luk-PV genes results in an attenuated agr system.” Specifically, similar RNAIII levels in the current data set (Figure 1B) suggest that there is virtually no effect of PVL on agr, given that RNAIII expression is directly correlated with agr functionality. This lack of consistency is in accordance with the results of our analysis, indicating that key experiments of Labandeira-Rey et al were performed with an agr-mutated strain. Thus, it is vital to routinely ascertain and reascertain the agr status of S. aureus strains used for key experiments.

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