A brief history of Staphylococcus aureus Panton Valentine leucocidin

Gerard Lina, François Vandenesch, and Jerome Etienne

INSERM, E0230, Lyon; Université Lyon 1, IFR62, Centre National de Référence des Staphylocoques, Faculté Laennec, 7 rue Guillaume Paradin, F-69008 Lyon, France

Correspondence to:
Jerome Etienne, Université de Lyon, INSERM E0230
Faculté de Médecine Laennec
Rue Guillaume Paradin
69372 Lyon Cedex 08. France
Phone: + 33 (0) 478 77 86 57 or 687 45 88 72
Fax: +33 (0) 478 77 86 58
Email: jetienne@univ-lyon1.fr

The history of Panton Valentine leucocidin (PVL), one of the toxins of *Staphylococcus aureus*, began in 1894, 24 years after Sir Alexander Ogston and Louis Pasteur had reproduced human pyemic infections in mice and rabbits by inoculating pus or pus-derived cultures (11,14). The torch of PVL research was then handed down over the decades between small groups of scientists fascinated by *S. aureus* toxins. Knowledge advanced in fits and starts, notably with the advent of new research tools.

The first studies focused on the effects of *S. aureus* on leukocytes, while the latest, 100 years on, involves genetic characterization of the leucocidin. In addition, highly epidemic PVL-positive methicillin-resistant *S. aureus* strains have recently emerged as a worldwide health issue, leading to a further upsurge of interest in PVL. In 1995, the English scientific literature contained only one title and six articles including the abbreviation PVL, compared to 36 titles and more than 200 articles in 2005.

1894. Van de Velde coins the term "leucocidin", based on the leukotoxic potential of some *S. aureus* strains

The first chapter in the staphylococcal leucocidin story was written by Dr Honoré Van de Velde, a physician working at the department of pathology and experimental medicine of Louvain University, Belgium. In 1894, Van de Velde was working on virulence determinants in *Staphylococcus aureus* and on the potential protective effect of leucocytes and "humor", an old word for serum. Most of his experiments were published in 1894 and 1895 (1,21).

To investigate *S. aureus* virulence, Van de Velde developed a model of pleural infection in rabbits and dogs. In rabbits, he found that leucocytes arrived in the pleural cavity an hour or two after local *S. aureus* injection. With the first isolate he injected, the leukocyte count increased gradually in the pleural cavity and the cells had a perfectly normal appearance. In contrast, another isolate seemed to kill the leukocytes that were recruited to the pleural cavity. Van de Velde wrote that the leukocytes had been "*shot down*". "*The cells were rounded, the nucleus condensed and the cytoplasm clarified, with no granules and no pseudopodes; then the cytoplasm disappeared. When the leukocytes were examined in a Zeiss warm chamber, they were totally immobile and showed no diapedesis. They had all been shot down.*"

This leukocidal activity was associated with 20-fold higher animal lethality and 1000-fold higher bacterial counts in the pleural cavity. Van de Velde deduced that bacteria with leukocidal activities were less readily destroyed by leukocytes. Then, by co-culturing leukocytes with *S. aureus*, he showed that "*only the isolate with leukocidal activity was able to grow at 37°C when leukocytes were added as early as 2 hours after the beginning of incubation. By contrast, the isolate without leukocidal activity was destroyed within 24 hours*". However, when pleural "*sérosité*" (supernatant) obtained from animals with pleural infection due to the leukotoxic isolate was added to co-cultures of non-leukotoxic *S. aureus* and leukocytes, the bacteria were able to grow as vigorously as their leukotoxic counterparts. Thus, Van de Velde argued that "*white blood cells are able to kill several bacteria, and their destruction should promote bacterial aggression. This would explain why bacterial leukotoxicity is associated with higher lethality for the animal*."

Van de Velde postulated that "*the leukotoxic isolate was able to produce a poison able to destroy leukocytes*." As this poison was inactivated by heating for 10 minutes at 58°C, it belonged to *the ferment family, and was an albuminoid (protein) molecule*. He proposed to call this poison *leukocidal substance*, or *leucocidin*. He suspected that leucocidin was a secreted product because, after centrifugation, staphylococcal supernatants were still highly toxic for white blood cells. Unfortunately, he did not mention whether or not his preparation still contained some bacteria.

Van de Velde was able to show that *S. aureus* produced the toxin not only in animals but also *in vitro*, by using blood, serum and broth cultures. He detected the leucocidin only after 48 hours of growth in these media, and concluded that "*the virulence of S. aureus is driven by its capacity to produce a poison able to put leukocytes out of action. Leucocidin production occurs only when staphylococci are abundant*."

1894. Van de Velde shows that dog leukocytes are resistant

Van de Velde also examined the effect of his isolates on dogs (21). In contrast to rabbit leukocytes, dog leukocytes were quite resistant to the leucocidin, suggesting that the leucocidin susceptibility of white blood cells was species-dependent.

1895. First attempts at leucocidin vaccination and serotherapy by Van de Velde

Finally, Van de Velde vaccinated rabbits with filtered supernatant, broth culture and heat-inactivated broth culture of leukotoxic *S. aureus* (1). Cachexia and skin infection occurred after vaccination with the broth culture. However, "*none of the vaccinated animals died when they were challenged by intra-pleural injection of the leukotoxic S. aureus isolates used to prepare the vaccine, except at very high doses (over 100 times more). Leukocytes recovered from the pleural cavity of vaccinated rabbits were perfectly normal. However, when they were separated from the pleural exsudate by sedimentation and treated with the leucocidin, they were destroyed. The addition of serum from normal rabbits did not have a protective effect. By contrast, when leukocytes were challenged with a mixture of leucocidin and serum from vaccinated rabbits, the leukocytes were perfectly normal*." By analogy with work by Behring and Kitasato on a diphtheria antitoxin (24), Van de Velde concluded that "*every vaccinated rabbit had acquired a serum with anti-leucocidin properties. The anti-leucocidin probably neutralizes the leucocidin by forming inactive complexes*."

1901. Neisser and Wechsberg clearly demonstrate that leucocidin is a secreted toxin

In 1901, M. Neisser and Wechsberg showed that a sterile filtrate of leukotoxic *S. aureus* was toxic for white blood cells (10). These authors were also the first to quantify the leukotoxic activity of staphylococcal supernatants, not only microscopically, but also by

measuring polymorphonuclear leukocyte respiratory activity in terms of methylene blue reduction in partially aerobic conditions.

1922. Julianelle distinguishes between leukotoxic and hemolytic activities

Staphylococci were rapidly shown to be toxic not only for leukocytes but also for other cells such as erythrocytes (the corresponding toxin was called a “hematotoxin”), and skin cells (dermatotoxin or necrotoxin). In Van de Velde’s manuscripts, they was no clear distinction between leukotoxic and hemolytic activities. When he incubated the leukotoxic *S. aureus* isolate with fresh blood from rabbits and dogs, he noted that "*the blood became darker and darker* and *finally the red blood cells were dissolved*". However, he attributed this to oxygen use by the bacteria and not to hemolysis. In his second publication he reported that red blood cells were altered by the leucocidin: "*red blood cells swelled and then dissolved*". Subsequent works focused on these staphylococcal extracellular substances possessing both specific and non specific toxic effects on leukocytes (10). It appeared that some staphylococcal hemolysins were also able to disrupt the structure and function of leukocyte membranes. Relationships were established between animal lethality, hemotoxin and necrotoxin (13). In retrospect, the leukotoxic properties of staphylococcal hemolysins hindered the characterization of the true staphylococcal leukocidin, and substances described as leucocidin, such as Neisser-Weschberg leucocidin, were eventually shown to be hemolysins (28).

It was only in 1922 that Louis A Juliannelle, from Philadelphia General Hospital, was able to distinguish between leukocidal and hemolytic activity (6). When he tested the biological properties of four different *S. aureus* strains grown in broth, he found that the supernatants of only two strains induced methylene blue reduction by guinea-pig leukocytes, reflecting their leukotoxicity. One of these strains did not produce a hemolysin, and a third strain, which produced most hemolysins, did not produce leucocidins. This "*distinctly indicated that hemolytic and leucocidin activities are not dependent on each other*".

1932. Panton and Valentine confirm that leucocidin is not associated with hemolytic activity and is produced by strains causing severe infections

Ten years later, Philip Panton and Francis Valentine, working at the Hale clinical laboratory of London Hospital, selected 22 *S. aureus* strains isolated from a variety of more or less severe human infections and compared their toxic properties (12). Hemolytic activity was tested on rabbit red cells, leukotoxicity on human leukocytes, necrosis by intradermal injection in rabbits, and lethality by intravenous injection to young rabbits. The authors observed no correlation between hemolytic and leukotoxic activites, indicating that leucocidin was distinct from hemolysins. Seven of the 22 isolates produced high leucocidin and low hemolysin activities, indicating that at least 30% of the selected isolates produced this true leucocidin. Moreover, when the authors examined toxin production according to the corresponding human infection, they noted that "*strains with strong leucocidin and weak hemolysin activity are commonly associated with acute severe infections, and the reverse combination is associated with long-standing superficial infection*". These severe infections consisted of four of skin and two of bone, complicated by pyemia or empyema.

Thus, they demonstrated that true leucocidin did not correspond to a hemolysin and that it was commonly associated with severe *S. aureus* infection.

1936. Valentine characterizes leucocidin-susceptible cells and shows an increase in the serum anti-leucocidin antibody titer during human infection

In 1936, in a second publication (20), Valentine showed that alpha-hemolysin was toxic for rabbit red and white blood cells, while the true leucocidin was capable of destroying all

phagocytic leukocytes both in human and rabbit blood, without affecting lymphocytes or red cells. Moreover, he noted that rabbit leukocytes destroyed by alpha-hemolysin generally looked different from human or rabbit cells destroyed by true leucocidin. "*Alpha-hemolysin-treated rabbit neutrophils appear less fragile; the nucleus is broken up but the granules cluster in one part of the cell. In contrast, cells treated with true leucocidin take on a spherical shape and the granules are mainly arranged at the periphery; in the presence of an excess of toxin, the cells burst and the granules are released*". These specific aspects were subsequently confirmed by other authors (17). While going through clinical records, Valentine also noticed that "*strains isolated from lesions in which real tissue invasion has occurred in an otherwise healthy patient nearly always produce considerable amounts of leucocidin*" and he also found a significant increase in serum anti-leucocidin activity during chronic superficial or deep-seated staphylococcal infections.

1936. Wright coins the term "Panton Valentine leucocidin"

This "true" staphylococcal leucocidin was first called Panton Valentine leucocidin in 1936 by J. Wright (28), who distinguished it from other staphylococcal products with leukotoxic activity.

1957. PVL detection in pyogenic infection

Further investigations at the Sir William Dunn School of Pathology of Oxford University, by G.P. Gladstone and W.E. van Heyningen, confirmed the selective action of Panton Valentine leucocidin (PVL) on granulocytes and macrophages, whereas staphylococcal alpha- and delta-hemolysins had multiple cytotoxic activities that included leukotoxicity (5). These researchers managed to produce large amounts of PVL by growing bacteria in modified CCY medium in rocking T-culture tubes -- a method still used to produce PVL. They also examined PVL production by a collection of staphylococcal coagulase-positive clinical isolates, by testing culture supernatants and the neutralizing effects of PVL antiserum. Thirty-one of 61 isolates from pyogenic infections produced PVL, compared to only two of 10 nasal colonization isolates. By contrast, no staphylococcal coagulase–negative saprophytic strains produced PVL. Thus, PVL production by *S. aureus* was quite frequent. How these isolates were selected is unclear.

1959. Purification of PVL, and characterization of its two components

In 1959, A.M. Woodin, from the same university, was the first to combine "modern" chemistry with leukocidal tests and immunology to characterize PVL from cultures in CCY medium (25). He was able to partially purify PVL by fractionation of the supernatant with ammonium sulfate and hydroxylapatite. He observed that "*on columns of cation-exchange resins, leucocidin separates into two main fractions*", which he called the 'fast' (F) and 'slow' (S) fractions. He wrote: "*There is loss of activity during the separation which is regained by mixing the fractions. The nitrogen contents, ultraviolet absorption and amploteric behaviour of the fractions of leucocidin are characteristic of proteins*", thereby establishing the dual nature of PVL. Woodin confirmed that the purified F and S components behaved immunologically as a single substance (26). Using purified components of PVL, Woodin archived a huge amount of data on PVL-leukocyte interactions. He summarized his painstaking work in several reviews, such as (27).

1962. Detection of anti-PVL antibodies in patients and development of a leucocidin antitoxin

Using purified components of PVL, Gladstone et al re-evaluated serum anti-PVL titers in infected individuals. He showed that anti-F and anti-S titers in 121 subjects free of clinical infections were more variable than the corresponding anti-alpha-hemolysin titers. Nineteen patients with osteomyelitis, furunculosis or chronic discharging abscesses had mean anti-F and anti-S titers 6 to 7 times the normal value. Unfortunately, the authors did not check whether the clinical isolates produced PVL (4). Gladstone subsequently developed a leucocidin toxoid by formolizing each purified component for 10 days, then precipitating and resolubilizing the two components in pyrogen-free saline. The leucocidin toxoid elicited specific antibodies in healthy subjects after a single intradermal injection. This leucocidin toxoid was administered therapeutically to patients with chronic staphylococcal osteomyelitis and soft-tissue infections (we do not know if the clinical isolates produced PVL). Highly significant increments in anti-F and anti-S leucocidin antibody titers were elicited within two weeks, and titers plateaued after 3 or 4 weeks. The authors speculated that "*patients, even with chronic suppurative lesions, were not making optimal immune response to PVL and that augmented response could be elicited by suitable inoculations*". "*Clinical impressions of cases were that the patient did appreciably better than would have been expected without immune therapy*". However, the toxoid preparation usually provoked local inflammation at the injection site (induration, erythema and soreness), and mild systemic reactions consisting of transient malaise and slight temperature elevation were also frequent.

1972 onwards. The genetic era: identification the phages encoding PVL, and characterization of the PVL genes

In 1972, Van der Vijver et al from the Department of Clinical Microbiology and Antimicrobial Therapy, Medical Faculty, Rotterdam (Netherlands) reported evidence of lysogenic conversion in *S. aureus* by a group A phage, leading to an increase in leucocidin production. However, the assay system they used failed to identify the precise type of leucocidin (22). Kamio's group at Tohoku University in Sendai (Japan) eventually confirmed that the PVL gene was carried by two phages, namely phiPVL and phiSLT (7,9). Kamio's group was also the first to describe genes encoding the F and S components of a leucocidin (18,19). Gilles Prévost et al from the Institut de Bactériologie of the Strasbourg Faculty of Medicine (France) showed that the PVL genes reported by the Kamio group corresponded to a variant of a gamma-hemolysin gene and themselves described PVL genes (*luk*-PV) (16). PVL genes consist of two co-transcribed open reading frames, *luk*S-PV and *luk*F-PV, coding for the class S and class F components of PVL.

1995. Distribution of PVL genes in clinical *S. aureus* isolates

Using rabbit antibodies raised against purified PVL components, Prévost et al used immunoprecipitation to determine the frequency of PVL production by human isolates (15). Only 5 of 309 consecutive isolates of *S. aureus* produced PVL: two from tracheal samples and three from unusually severe skin lesions. When they analyzed the distribution of PVL-producing strains among patients with cutaneous infections or septicemia and in asymptomatic nasal carriers, they found that PVL production was associated with skin infections, and especially furuncles (15). Yves Piémont, who supervised this work, was an expert on toxin detection and regularly received French strains of *S. aureus* isolated from patients with unusual clinical features. He was struck by the strong association between PVL-producing strains and furuncles and, conversely, the association of PVL with rapidly fatal staphylococcal infections in immunocompetent children. Fatal outcome was associated with leukopenia and hemoptysis, usually preceded by a simple influenza-like syndrome. This prompted Piémont to contact Jerome Etienne, as Director of the French National Center for Staphylococcal Diseases in Lyon, to

confirm and refine these results by screening our collection for PVL. The first results of this investigation were published in 1999 by Lina et al, who used PCR to screen 172 *S. aureus* isolates for *luk*-PV and confirmed the strong association of PVL-positive *S. aureus* with furunculosis, but also with community-acquired pneumonia, that was fatal in 14 out of 23 cases (8).

2002. Description of necrotizing pneumonia associated with PVL-positive *S. aureus* isolates, and detection of PVL genes in community-acquired *S. aureus* isolates

Pneumonia due to PVL-positive *S. aureus* strains was investigated by Gillet et al in 2002, by comparison with PVL-negative *S. aureus* pneumonia (3). PVL-producing *S. aureus* strains caused hemorrhagic, necrotizing pneumonia that rapidly progressed towards an acute respiratory distress syndrome with hemoptysis and leukopenia, usually in otherwise healthy children and young adults. It was often preceded by an influenza-like syndrome and killed three-quarters of its victims. Autopsy showed bilateral pulmonary hemorrhaging and necrosis. Many other cases have since been reported worldwide.

In 2002, Dufour et al detected PVL genes in methicillin-resistant *S. aureus* strains isolated from French patients with community-acquired infections (2). In 2003, Vandenesch et al detected PVL genes in methicillin-resistant *S. aureus* clones from Europe, North America and Oceania (23). This discovery of PVL genes in highly epidemic clones circulating in the community has further focused the minds of the scientific community on this devastating toxin.

Conclusion

Work on PVL began in 1894, but the story is far from over. The precise role of PVL in the pathogenesis of severe *S. aureus* infections is still not known. The incidence of PVL-associated *S. aureus* infections will continue to increase in coming years as community methicillin-resistant *S. aureus* strains bearing the PVL genes continue to spread worldwide. Work continues on the "anti-toxin" or "toxoid" concept of treatment and prophylaxis.

REFERENCES

1. Denys J, Van de Velde H. Sur la production d'une antileucocidine chez les lapin vaccinés contre le staphylocoque pyogène. La Cellule 1895;11:359-72.

2. Dufour P, Gillet Y, Bes M, Lina G, Vandenesch F, Floret D, Etienne J, Richet H. Community-acquired methicillin-resistant *Staphylococcus aureus* infections in France: emergence of a single clone that produces Panton-Valentine leukocidin. Clin Infect Dis 2002;35:819-824.

3. Gillet Y, Issartel B, Vanhems P, Fournet JC, Lina G, Bes M, Vandenesch F, Piemont Y, Brousse N, Floret D, Etienne J. Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet 2002;359:753-759.

4. Gladstone GP, Mudd S, Hochstein HD, Lenhart NA. The assay of anti-staphylococcal leucocidal components (F and S) in human serum. Brit J Exp Path 1962;43:295-312.

5. Gladstone GP, van Heyningen WE. Staphylococcal leucocidins. Br J Exp Pathol 1957;38:123-37.

6. Julanielle LA. Studies of hemolytic staphylococci. Hemolytic activities, biochemical reactions, serological reactions. J Infect Dis 1922;lxii:256-84.

7. Kaneko J, Kimura T, Narita S, Tomita T, Kamio Y. Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage phiPVL carrying Panton-Valentine leukocidin genes. Gene 1998;215:57-67.

8. Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V, Vandenesch F, Etienne J. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis 1999;29:1128-32.

9. Narita S, Kaneko J, Chiba J, Piemont Y, Jarraud S, Etienne J, Kamio Y. Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, phiSLT. Gene 2001;268:195-206.

10. Neisser M, Wechsberg F. Über das Staphylotoxin. Z Hyg Infektkrankh 1901;36:299-349.

11. Ogston A. Über Abscesse. Arch Klin Chir 1880;25:588-600.

12. Panton NP, Valentine FCO. Staphylococcal toxin. Lancet 1932;222:506-8.

13. Parker Weld JT, Gunther A. Differentiation between certain toxic properties of filtrates of hemolytic *Staphylococcus aureus*. J Exp Med 1931;liv:315-22.

14. Pasteur L. De l'extension de la théorie des germes à l'étiologie de queques maladies communes. C.R. Séances Soc Biol Paris 1880;90:1035-44.

15. Prévost G, Couppier P, Prévost P, Gayet S, Petiau P, Cribier B, Monteil H, Piemont Y. Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. J Med Microbiol 1995;42:237-45.

16. Prévost G, Cribier B, Couppié P, Petiau P, Supersac G, Finck-Barbancon V, et al. Panton-Valentine leucocidin and gamma-hemolysin from *Staphylococcus aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. Infect Immun 1995;63:4121-9.

17. Proom H. The inter-relationships of staphylococcal leucocidins. J Path Bact 1937;44:425-9.

18. Rahman A, Izaki K, Kato I, Kamio Y. Nucleotide sequence of leukocidin S-component gene (lukS) from methicillin resistant *Staphylococcus aureus*. Biochem Biophys Res Commun 1991;181:138-44.

19. Rahman A, Nariya H, Izaki K, Kato I, Kamio Y. Molecular cloning and nucleotide sequence of leukocidin F-component gene (lukF) from methicillin resistant *Staphylococcus aureus*. Biochem Biophys Res Commun 1992;184:640-6.

20. Valentine FCO. Further observations on the role of the toxin in staphylococcal infections. Lancet 1936;i:526-31.

21. Van de Velde H. Etude sur le mécanisme de la virulence du staphylocoque pyogène. La Cellule 1894;10:403-60.

22. Van der Vijver JC, Van Es-Boon M, Michel MF. Lysogenic conversion in *Staphylococcus aureus* to leucocidin production. J Virol 1972;10:318-9.

23. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, Liassine N, Bes M, Greenland T, Reverdy ME, Etienne J. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 2003;9:978-84.

24. Von Behring E, Kitasato S. Ueber das Zustandekommen der Diphtheria-Immunität und der Tetanus-Immunität. Dtsch Med Wochenschr 1890;16:1113–4.

25. Woodin AM. Fractionation of a leucocidin from *Staphylococcus aureus*. Biochem J 1959;73:225-37.

26. Woodin AM. Purification of the two components of leucocidin from *Staphylococcus aureus*. Biochem J 1960;75:158-65.

27. Woodin AM. Staphylococcal leucocidin. In: Cohen JO, editor. The staphylococci. Ney York, NY: Wiley-Interscience; 1972. p. 281-99.

28. Wright J. Staphylococcal leucocidin (Neisser-Wechsberg type) and antileucociddin. Lancet 1936;230:1002-4.