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Research Article

Site-Directed Mutagenesis to Assess the Binding Capacity of Class S Protein of *Staphylococcus aureus* Leucotoxins to the Surface of Polymorphonuclear Cells

L. Baba Moussa,[1] S. Werner,[2] M. Coraiola,[3] D. A. Colin,[2] D. Keller,[2] A. Sanni,[1] M. Dalla Serra,[3] H. Monteil,[2] and G. Prévost[2]

[1] *Département de Biochimie et de Biologie Moléculaire, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, BP 04-0320, Cotonou, Benin*
[2] *Laboratoire de Physiopathologie et d'Antibiologie Bactériennes des Infections Emergentes et Nosocomiales, UPRES EA 3432, Institut de Bactériologie de la Faculté de Médecine de Strasbourg, Hôpitaux Universitaires de Strasbourg, Université Louis Pasteur, 3 rue Koeberlé, 67000 Strasbourg, France*
[3] *CNR—ITC, Istituto di BioFisica, Università di Trento, Via Sommarive 18 38050 Trento, Italy*

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Staphylococcal leucotoxins result from the association of class S components and class F component inducing the activation and the permeabilization of the target cells. Like α-toxin, the leucotoxins are pore-forming toxins with more than 70% β-sheet. This was confirmed by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. In addition, threonine 28 of a predicted and conserved β-sheet at the N-terminal extremity of class S proteins composing leucotoxins aligns with histidine 35 of α-toxin, which has a key role in oligomerization of the final pore. Flow cytometry was used to study different aminoacid substitutions of the threonine 28 in order to evaluate its role in the biological activity of these class S proteins. Finally, results show that threonine 28 of the leucotoxin probably plays a role similar to that of histidine 35 of α-toxin. Mutations on this threonin largely influenced the secondary interaction of the class F component and led to inactive toxin.

INTRODUCTION

Staphylococcus aureus (S aureus) is one of the most frequently isolated bacterium in hospital routine, fearing infections that may affect any organs and tissues. Having developed resistance to most of antimicrobials, it is now responsible for 5–15% of nosocomial infections, depending on hospital sites and services [1]. The pathogenicity of this bacterium is caused by a series of adhesion factors [2] and toxins. Among these toxins, staphylococcal leucotoxins are a family of bicomponents toxins [3] that result from the association of class S and class F components that interact sequentially and synergistically [4], inducing the activation and the permeabilization of the target cells. The class S protein first binds to the membrane of target cells and then allows the secondary binding of class F component. These toxins target polymorphonuclear cells (PMNs), monocytes, macrophages, and erythrocytes [5, 6]. Among this family of toxins, Panton-Valentine leucocidin (LukS-PV + LukF-PV) and gamma-hemolysin, which generates two toxins (HlgA + HlgB and HlgC + HlgB), activate response of specific cells via a Ca^{2+} influx and form lethal transmembrane pores. LukS-PV, HlgA, and HlgC are class S components, while LukF-PV and HlgB are class F components (Figure 1). The genes encoding these toxins have been cloned and sequenced [7–10]. Sequence homologies are very important inside the two classes of proteins. Identities are up to 55–70% for class S and 70–80% for class F proteins, but only 18–25% between the two classes [11, 12]. Additional homologies exist between the two classes of proteins and other pore-forming toxins such as with staphylococcal α-toxin [13]. Like α-toxin of *S aureus*, leucotoxins are pore-forming toxins with predominant β-sheet [13]. By aligning leucotoxins and α-toxin (Figure 2), Thr28 appears preserved into a predicted β-sheet at the N-terminal extremity of the leucotoxins and corresponds to His35 of α-toxin [14]. His35 has a role in the protein oligomerization and plays a critical role in its function [15]. The aim of this work was to study the functional tolerance

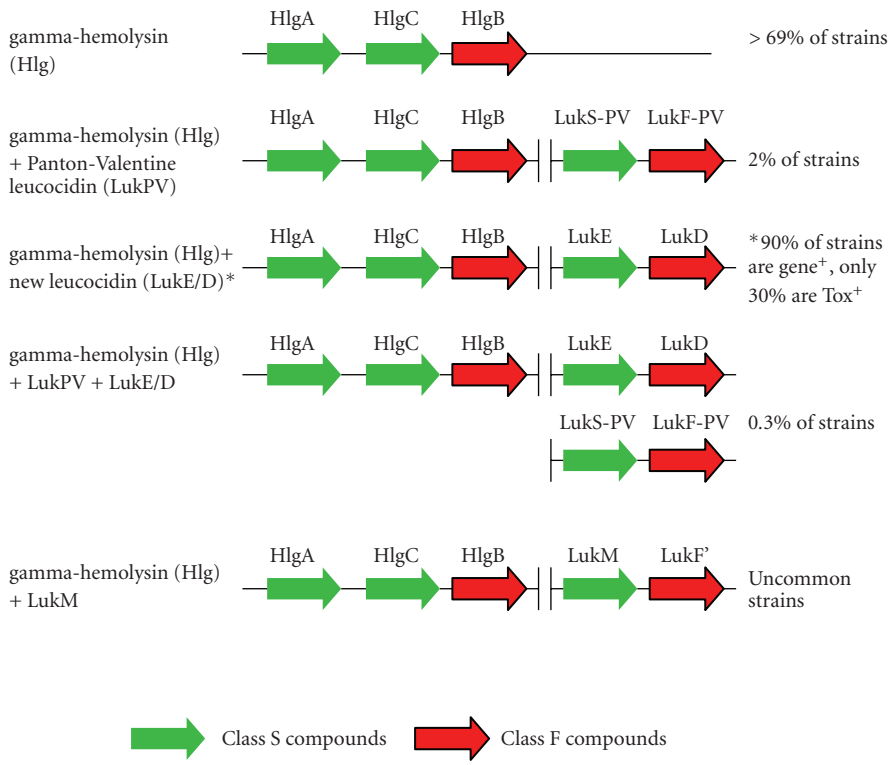

Figure 1: Distribution of the genes expressing leucotoxins of *S aureus.*

α-toxin	31	E N G M **H** K K V F Y S F I D D K
LukE	25	W – G V **T** Q N V Q F D F V K D K
LukS-PV	25	W – G V **T** Q N I Q F D F V K D K
HlgA	25	L – A I **T** Q N I Q F D F V K D K
HlgC	27	W – G V **T** Q N I Q F D F V K D K
LukM	25	W – G V **T** Q N V Q F D F V K D K

Figure 2: Partial alignment α-toxin sequence and class S component sequence of the leucotoxins.

by leucotoxins of different substitutions of threonine 28 of a class S protein. Some of the mutations were predicted to disrupt the second β-sheet at the N-terminal extremity. The secondary structure alteration of the purified mutated proteins was confirmed by Fourier transform infrared spectroscopy, and their biologicals activities were evaluated by flow cytometry onto human circulating PMNs and rabbit erythrocytes.

MATERIALS AND METHODS

Cloning and sequencing of the mutated proteins

DNA fragments corresponding to secreted proteins and containing the putative 3′ inverted repeats were amplified via dedicated oligonucleotides containing *EcoR*1 restriction sequences at their 5′-end. After a further *EcoR*1 restriction, the amplified DNA fragments were cloned in the *EcoR*1-dephosphorylated pGEX-6P-1 (Amersham Biosciences, Orsay, France) expression vector. The correctly oriented recombinant plasmids were used as templates for any of the mutations discussed in this work. Site-directed mutagenesis was achieved by using the Quick Change Mutagenesis kit (Stratagene, Amsterdam, The Netherlands). Briefly, reactions were performed in the presence of 5 ng of template (5.8 kb), 0.25 mM dNTP, 0.4 nM of each dedicated oligonucleotide, and 5 U of Pfu Turbo DNA polymerase in 50 μL. Temperatures of hybridization, elongation, and denaturation were 50°C, 68°C, and 95°C during 30 seconds, 3 minutes, 30 seconds, and 1 minute, respectively. Initial templates were eliminated with an 80 minutes *Dpn*I restriction, and 2.5 μL of the resulting mixture was used for transformation of 80 μL of XL1 Blue *E coli* XL1 Supercompetent Blue cells [*rec*A1 *end*A1 *gyr*A96 thi1 *hsd*R17 *supE*44 *rel*A1 *lac* (F′ *pro*AB *lac*I^q *Z*ΔM15 *Tn*10 (tet^r))]. Mutated genes were sequenced from Plasmid Midi kit preparations Qiagen (Paris, France), and plasmids only containing the mutation were electroporated at 1.8 kV, 200 Ohms, 25 μFd in 80 μl of *E coli* BL21 [F-, *omp*T, *hsd*S (*rB*$^-$, *mB*$^-$), *gal* (52, 53)] (Amersham Biosciences, Orsay, France) previously stored at −80°C at 2.0 $A_{600\,nm}$ units in 0.1 mM Hepes, pH 7.0. Electroporated cells were regenerated in SOC medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$,

20 mM glucose, pH 7.0) for 1 hour at 37°C. Transformed bacteria were finally treated and plated as recommended (GST Gene Fusion System (Amersham Biosciences, Orsay, France)).

PURIFICATION OF *E COLI* RECOMBINANT LEUCOTOXIN COMPONENTS

The mutated proteins were purified as glutathione-S-transferase-(GST) fusioned leucotoxins. Recombinant *E coli* BL 21 mutated clones were inoculated from a starter culture into 2×400 mL of TY medium filled in two-liter Erlenmeyer flasks, and cultivated for 6 hours before overnight induction of the GST-fusioned protein with 0.2 mM IPTG. Bacteria were harvested by centrifugation and concentrated to 30% (w/v) into 30 mM NaH_2PO_4, 150 mM NaCl, and 1 mM EDTA, pH 7.0. Then, bacteria were disrupted at 9000 psi with a French pressure press (SLM Instruments, Ill, USA; Bioritech, Joinville, Juine, France). Cell debris were discarded by a 30-minute centrifugation at $30\,000 \times g$ at 6°C, and GST activity was measured at 340 nm as recommended. A volume of lysate equivalent to 4 mg of titrated GST was applied onto a Glutathione Sepharose 4B (Pharmacia) column equilibrated with 60 mM Tris-HCl, pH 8.0. The fusion protein was eluted in the same buffer containing 30 mM glutathion, and materials were further digested overnight by 5 U of PreScission protease (Pharmacia) per mg of eluted proteins. The leucotoxin components were purified through a 1.35 M to 0.45 M $(NH_4)_2SO_4$ gradient applied on an alkylsuperose fast performance liquid chromatography (Pharmacia). The proteins were eluted at 0.75 M $(NH_4)_2SO_4$. The pre-purified F components were dialyzed against 30 mM MES, pH 6.3, and purified to homogeneity by a MonoS FPLC through a 0 to 150 mM NaCl gradient, with elution around 90 mM NaCl. Purified proteins were controlled by SDS-PAGE and radial gel immunoprecipitation and stored at $A_{280\,nm}$=1.0 at −80°C.

Preparation of human polymorphonuclear cells

Twelve milliliters of J-Prep solution (TechGen, Les Ulis, France) were added to 30 mL of buffy coats from healthy donors diluted with 10 mL of 0.9% (w/v) NaCl, and centrifuged for 20 minutes at $800 \times g$ at room temperature. The cell pellet was suspended in 40 mL of 0.9% (w/v) NaCl, 1.5% (w/v) dextran and left to sedimentation for 30 minutes. The supernatant was carefully removed and then centrifuged at room temperature at $800 \times g$ for 10 minutes. The new supernatant was discarded and the erythrocytes pellet containing PMNs was resuspended and lysed into 18 mL of apyrogenic water for 45 seconds, before complementing the suspension with 2 mL of 9% (w/v) NaCl. After two washing steps in 50 mL of 140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.1 mM ethylene glycol-bis (beta-aminoethyl ether) N, N, N′, N′-tetraacetic acid, 10 mM Hepes, and 3 mM Tris Base (pH 7.3), the cells were suspended and adjusted to 2×10^6 cells/mL in the same buffer. The latter buffer was used to wash the cells treated with the Ca^{2+}-specific fluorescent probe Fluo-4 (Molecular Probes, Eugene, Oregon, USA).

Labelled proteins

LukS-PV G10C, a functional cystein mutated protein [16], was labelled with fluorescein 5-maleimide (Molecular Probes, Lerden, The Netherlands) at a 5-fold excess for a 10 μM protein solution for 30 minutes at room temperature in 50 mM Na_2HPO_4, 0.15 M NaCl, 1 mM EDTA, and Na_2, pH 7.0. The coupling reaction was stopped by the addition of 10 mM β-mercaptoethanol. The mixture was then desalted, and the coupling yield was determined by the ratio of the determined concentration of fluorescein ($\varepsilon_{490\,nm} = 81,900\,cm^{-1} \cdot mol^{-1}$) and that of the protein determined by the Bradford's titration (Biorad, Ivry, Seine, France).

Flow cytometry measurements

Experiments were made with 5×10^5 cells/mL loaded in 5 μM Fluo-4 solutions during 1 hour at 37°C, then washed and resuspended in the presence of 1.1 mM Ca^{2+} for the evaluation of the Ca^{2+} entry. Each component constituting the leucotoxins was generally added at 1 nM. Flow cytometry measurements were performed by using a FacSort cytometer (Becton Dickinson, Le Pont de Claix, France) equipped with a 15 mW argon laser tuned at 488 nm. Forward (FSC) and side (SSC) light scatter dot plots acquired from 3000 purified leucocytes were classically gated [14, 17]. Fluo-4 fluorescence due to the calcium penetration was recorded from the fluorescence light 1 (FL1: $\lambda_{Em} = 530$ nm) every 30 seconds during 45 minutes. After basic fluorescence subtraction, results were compared to the maximum fluorescence obtained with controls which indicated the potentiality of the different pairs of leucotoxins for the opening of Ca^{2+} channels. The fluorescence light 3 (FL3: $\lambda_{Em} = 650$ nm) was used to record the fluorescence of ethidium applied at 25 nM together with the leucotoxins. This fluorescence increased when the molecule entered the cells by the pores formed through the plasma membrane and combined with nucleic acids. Means from four series of significant data, at least, obtained from PMNs of four or more different donors were calculated by Lysis 2TM software (Becton Dickinson), and the results were expressed as regression curves by using SigmaPlot facilities as mean percentages of maximum fluorescence detected from controls. Standard deviations were not shown for clarity of the figures, but did not vary more than ±6% as determined by SigmaPlot 8.0 resource.

By using a full functional fluorescein-labelled LukS-PV G10C, the binding abilities of LukS-PV and inactive mutants combined with 3 nM LukF-PV were determined by competition experiments in the absence of extracellular calcium, using a fixed concentration of 20 nM of the labelled LukS-PV G10C and variable concentrations of 1, 5, 10, 20, 50, 100, and 200 nM of the mutated proteins. The residual fluorescence at the cell surface was gated during 50 minutes after the application of the protein couples X + LukF-PV, in order

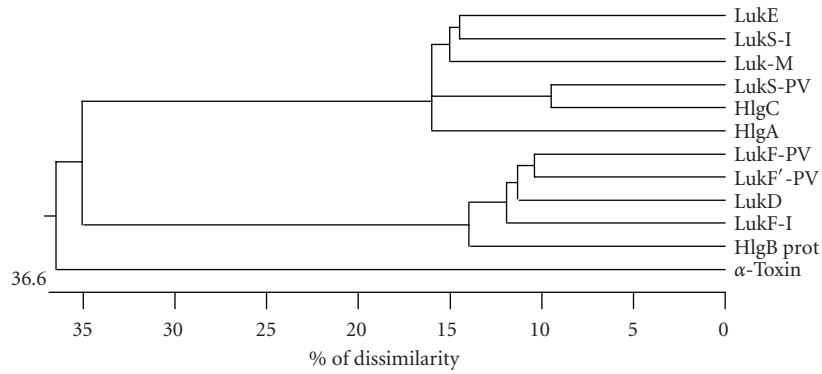

Figure 3: Philogenetic tree of peptidic homologies between the leucotoxins and α-toxin of *S aureus*. Unit = Euclidian distance (DNA Star, Ltd, London).

to minimize the influence of lysed cells in the data. Apparent inhibition constants (k_{iapp}) were deduced from projection to abscise the 50% means fluorescence values (IC50), with the following equation:

$$k_{\text{iapp}} = IC50/1 + [F]/Kd_F, \quad (1)$$

where [F] is the concentration of the fluorescent LukF-PV LukS-PV Gly10Cys and $K_{dF} = 3$ nM [18].

Secondary structure determination by ATR-FTIR

ATR-FTIR spectra were recorded on Biorad FTS 185 FTIR spectrometer equipped with DTGS detector with CSL window, a KBr beamspliter, and an ATR attachment by Specac. Typically 32 interferograms were collected, Fourier transformed to a nominal resolution of 0.5 cm^{-1}, and averaged. The instrument was constantly purged with dry air. Spectra were corrected by a subtracted background obtained with an ATR crystal and no sample. The residual absorbance of H_20 was subtracted to give an almost flat baseline between 1880 and 1720 cm^{-1}. Before analysis, the leukotoxins were extensively dialyzed against 10 mM Hepes (pH 7.0). For the experiments, 28 to 40 μg of each protein contained in 40 μL of the given leukotoxin solution were deposited and dried in thin layer on one side of a 10 reflections Ge crystal (45°C cut): the crystal was housed in liquid cell and flushed with D_2O-saturated nitrogen for 45 minutes before collecting the reported spectra.

The ATR-FTIR spectra were processed using the Biorad Win-IR software. Spectra were dissolved to 2 cm^{-1}, and the amide I′ band, between 1600 and 1700 cm^{-1}, was curve-fitted with a sum of Lorentzians, using nonlinear least squares fitting of Levenberg-Marquadt method. No parameter was constrained. The relative contents of secondary structure elements were estimated by dividing the area of individual peak, assigned to particular secondary structures, according to Byler and Susi [19], by the area of the whole amide I′ band; the components around 1600 cm^{-1}, resulting from the side chains, were excluded.

RESULTS

Secondary structure prediction

The secondary structure prediction for class S and class F proteins reveals significant homologies, as expected for proteins harbouring from 60 to 98% of sequence identity [20]. The sequence alignment by the software DNAStar of α-toxins and two component leucotoxins of class S shows that their sequence identity is less than 26% (Figure 2), but remained compatible with a common ancestor as shown on the philogenetic tree (Figure 3). These structural homologies have been unambiguously confirmed by the determination of three-dimensional structures of LukF-PV [21], and LukS-PV [22]. Thr28 in the class S of two component leucotoxins aligns with His35 of α-toxin (Figure 2). As an attempt to verify whether threonine residue of these class S leucotoxins has the same role as this His35, amino acid substitutions were introduced that would predictably disrupt the β-sheet of these class S proteins, which is highly conserved in the N-terminus region (Chou and Fassman, and Kite and Doolittle programs). Ten mutations were performed, eight on LukS-PV (LukS-PV T28D, LukS-PV T28N, LukS-PV T28N+ΔK43, LukS-PV T28F, LukS-PV T28L, LukS-PV N30T, LukS-PV D34C, LukS-PV D34S), one on HlgA (HlgA T28D), and one on HlgC (HlgC T30D). The purified proteins showed apparent molecular masses comparable with the staphylococcal native proteins (data not shown). Substitutions of Thr28 by Asp in HlgA and HlgC induced a strong decrease in β-structure (Table 1) up to 12.5%. In the case of HlgA T28D, such decrease is compensated by an increase in β-turn structure, whereas in the case of HlgC T30D, there is an increase in the unordered structure. For LukS-PV, there is a decrease in β-structure for all mutations (less than 3%) except for LukS-PV T28L (6.5%), but these decreases remain low compared to HlgA and HlgC mutants (Table 1).

Binding capabilities of the mutated proteins

The binding capability of the mutated protein LukS-PV G10C was first tested on polymorphonuclear, monocytes,

Table 1: Determination of the secondary structure of the leucotoxins by ATR-FTIR. The Lorentzian average corresponds to particular secondary structure (Byler and Susi). β_1 = antiparallel β-sheet, β_2 = parallel and antiparallel β-sheet, α = α-helice, t = β-turn and r = unorganized structure. $\beta_{\text{total}} = \beta_1 + \beta_2$. The errors following the independent testing are ± 5%.

Proteins	βt	t	α	r	β	β total
HlgA	6.7	14	15	15	57	63
HlgA T28D	9.6	25	4	17	43	53
HlgC	4.3	21	11	5	58	62
HlgC T30D	4.7	24	11	14	45	49
LukS-PV	10	49	10	8	20	60
LukS-PV T28D	9	46	12	10	20	56
LukS-PV T28N	8	48	12	10	20	56
LukS-PV T28N +ΔK	9	48	14	11	17	57
LuksPV T28F	10	45	12	9	21	56
LuksPV T28L	7	46	11	12	22	53
LukS-PV N30T	9	50	12	11	17	59
LukS-PV D34C	6	53	13	13	13	56
LukS-PV D34S	9	47	13	11	18	57

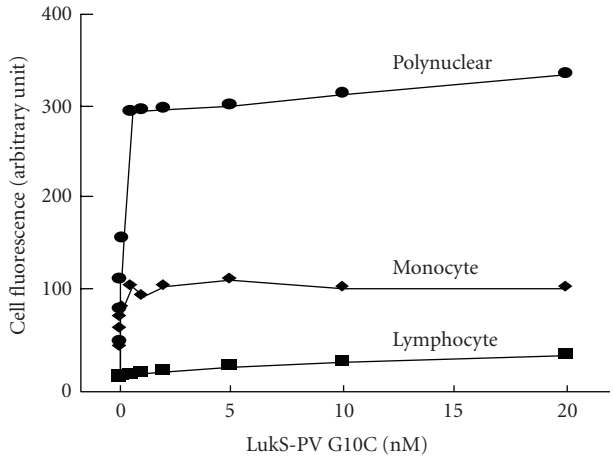

Figure 4: Determination by flow cytometry of LukS-PV G10C fluorescein-labelled association constant (λex = 488 nm, λem = 5 nm) with the human monocytes, polynuclears, and lymphocytes (2×10^5 cell/mL). Polynuclear: K_D = 0.13 nM; monocyte: K_D = 0.04. The LukS-PV Concentration is in nM.

and lymphocytes. Figure 4 shows that the fluorescein-labelled LukS-PV G10C is able to bind correctly to the target cells (k_{iapp} = 0.07 nM). The binding on lymphocytes was not detectable. The fluorescent mutated LukS-PV G10C is functional (see below).

In order to determine whether the mutated LukS-PV proteins are able to bind to the polymorphonuclear, a binding competition was performed between these mutated proteins and the fluorescein-labelled LukS-PV G10C. Figure 5 shows a similar binding of all the mutated proteins in competition with the fluorescent LukS-PV G10C, except for LukS-PV T28L for which a k_{iapp} could not be determined. The apparent inhibition constants (k_{iapp}) of these proteins are comparable to the estimated dissociation constant (K_D) of the native protein (Figure 5). Another kind of binding competition was performed between each LukS-PV mutated protein and the class F component HlgB labelled with 5-[4, 6-dichlorotriazin-2-YL] amino-fluorescein (DTAF). Figure 6 shows that only the native LukS-PV allowed the secondary binding of HlgB-DTAF. When combined with the mutated protein HlgB-DTAF, LukS-PV mutants are not able to bind to the cell membrane (Figure 6).

Calcium and ethidium entries induced by LukS-PV mutated proteins

Calcium and ethidium entries were measured by flow cytometry. The calcium entry was measured in the presence of 1 nM extracellular calcium and the ethidium entry was measured in the absence of calcium. Figure 7(a) shows that the native LukS-PV protein allows a strong entry of Ca^{2+} and an entry of ethidium. The entry of calcium induced by the mutated proteins is low. Two mutants, LukSPV T28L and LukS-PV T28N, displayed an entry of calcium. For the entry of ethidium, five mutants: LuksPV T28L, LukS-PV T28N, LukS-PV T28N+ΔK43, LukS-P T28D, and LukS-PV D34S, displayed an entry of ethidium (Figures 7(b) and 7(c)), while three mutated proteins: LukS-PV D34T, LukS-PV D34C, and LukS-PV N30T, were as active as the native protein.

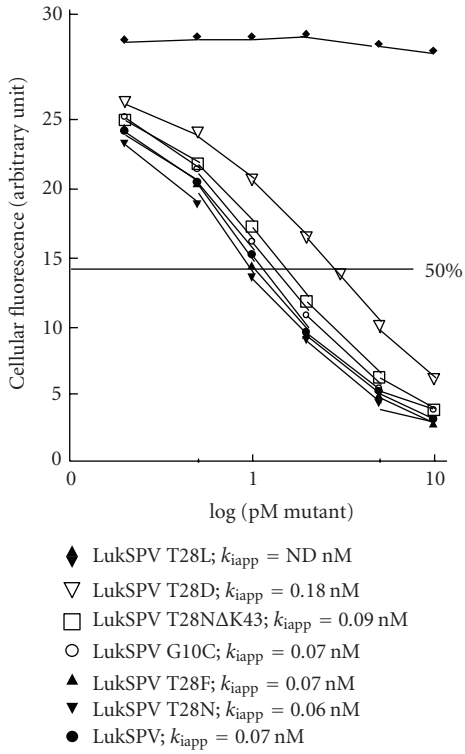

Figure 5: Competition between the fluorescent LukS-PV G10C and the mutated proteins and their binding to the cell, recorded by flow cytometry.

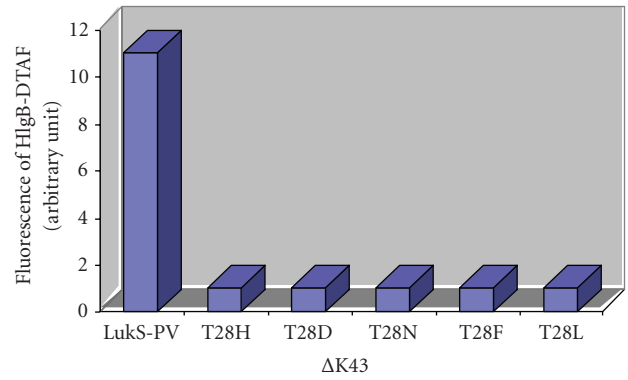

Figure 6: Determination of the secondary binding of a class F component mutated protein onto human PMNs. The determination of the binding is made by measuring the fluorescence of HlgB-fluorescein (0.75 nM) on bound mutated class S proteins (2 nM).

DISCUSSION

This work shows that the Thr28 of class S component of bicomponent leucotoxins may have a role in the whole structure because its substitution into a Leucine is responsible for a dramatic loss of the resulting LukS-PV. However, the Thr28 residue is a pore directly involved in the secondary binding and, may be, interaction with class F component. The decrease in the fluorescence of the native LukS-PV G10C (Figure 5) shows that there is a competition with the mutated protein. All the mutants have an affinity comparable to wild-type proteins (k_{iapp}~0.5 nM) for LukS-PV receptor except T28L which seemed devoid of binding. The substitution of Thr28 by different amino acids (Asn(N), Asp(D), Phe(P), Leu(L), Asn(N) ΔLys(K) 43) induced a decrease in β-structure up to 12.5% and had an influence on the secondary interaction of class F components, thus leading to inactive toxins. The mutations introduced in the class S proteins on Thr28 never prevent the binding of these proteins on their membrane ligand, that is, a receptor for LukS-PV [18], but hindered the secondary binding of class F component onto human PMNs. At this stage, Ca^{2+}-activation and the pore-forming function of the bicomponent leucotoxins were both not possible despite their uncoupling remained possible [16, 23]. The sequence identity between α-toxin and the bipartite leucotoxins is less than 26%, but remains compatible with the existence of a common ancestor when amino acids, homologies, and structures are considered. Thr28 of leucotoxins, as His35 of α-toxin, is probably an amino acid essential for oligomerization and for biological activity. Other substitutions, N30T and D34S, also affected the toxin activity suggesting that other residues than Thr28 may be involved in the interaction between the two subunits constituting leucotoxins. However, although the leucotoxins are members of the superfamily of β-barrel pore-forming toxins, they differ from similar toxins like α-toxin in some aspects. Their binding onto PMN membranes could be accompanied by the activation of a receptor, while these cells are not sensitive to α-toxin [18]. On one hand, pores formed by bipartite leucotoxins are more selective to monovalent cations (Na+, K+, ethidium) [24]. Oligomerization of bicomponent leucotoxins may involve residues at similar positions as for α-toxin, but one amino acid Tyr101 of α-toxin aligns with Tyr99 of F components of leucotoxin, and Tyr99 on HlgB or LukF-PV structures does not look accessible [21, 25]. In conclusion, the substitutions made on Thr28 point out its key role in the oligomerization and the function of the staphylococcal leucotoxins. This observation confirmed a previous study which showed that when Thr28 of HlgA (or the corresponding Thr30 of HlgC) was substituted by Asp, the mutants were still able to bind target cells and compete with the wild type proteins, but the subsequent binding of HlgB was abolished, with a complete loss of biological activity [14]. This was observed with human PMNs, erythrocytes, and PC-Cho small unilamellar vesicles. Thr28 of HlgA aligns with His35 of α-toxin, a crucial residue for monomer-monomer interactions [15, 26]. As leucotoxins are naturally devoid of cysteines into their structure, mutagenesis approach might be interesting to stabilize heterodimers of leucotoxins in order to assess the amino acids involved in these interactions between S and F proteins of bipartite leucotoxins. The determination of the three-dimensional structures of LukF-PV [21] and recently for LukS-PV [22] now enables to challenge some residues in these interactions.

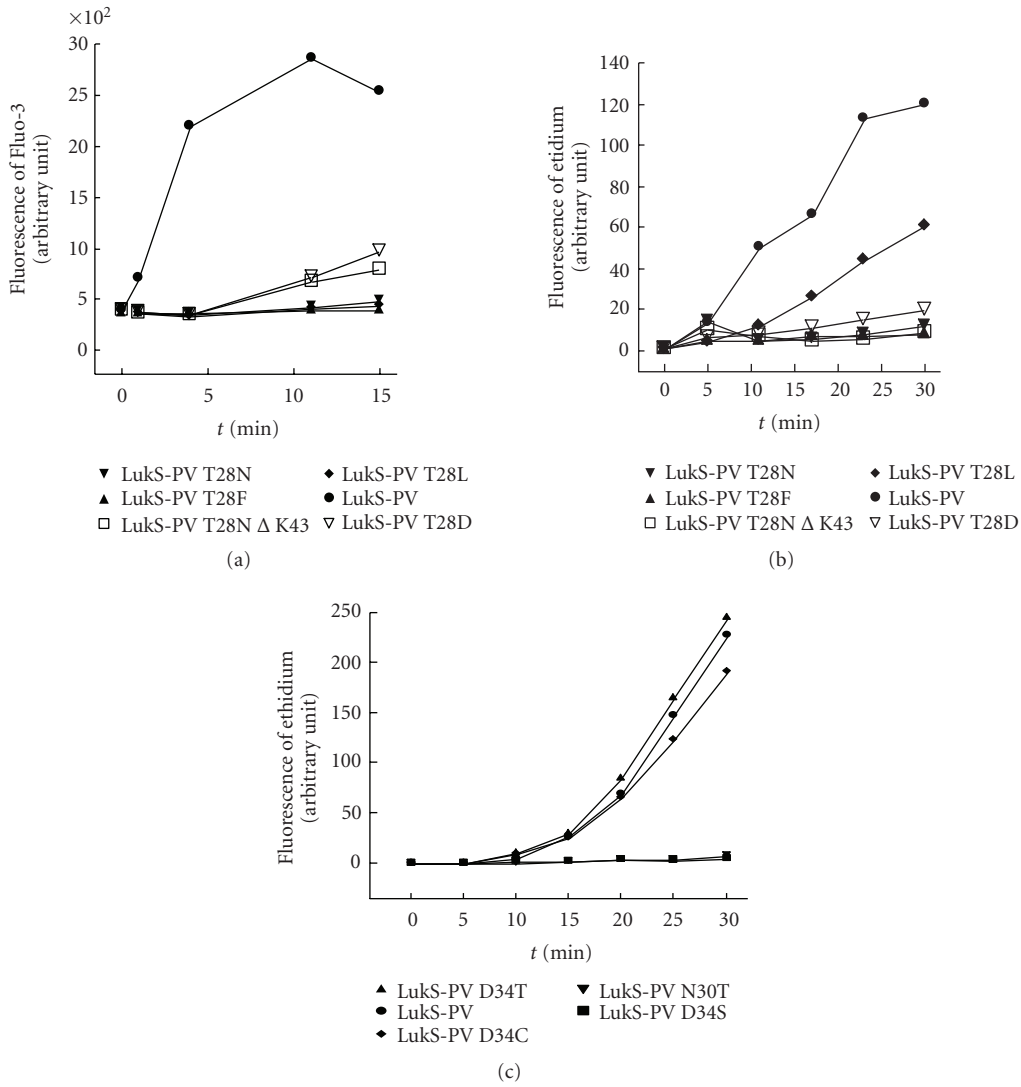

Figure 7: Determination by flow cytometry of (a) the opening of calcium channel in the presence of 1 mM of calcium by the measurement of the fluorescence intensity of Fluo-3 (λex = 488 nm, λem = 530 nm) and (b), (c) the formation of the pores into membranes in absence of calcium by the measuring of the fluorescence intensity of ethidium (λex = 488 nm, λem = 650 nm) for LukS-PV and the mutated proteins (2 nM) associated to LukF-PV (0.75 nM) on the membrane of human PMNs (2×105 cells/mL).

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