FEBS Letters 436 (1998) 202–208 FEBS 20890

Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family

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Received 19 August 1998

Abstract A new member of the staphylococcal bi-component leucotoxins family, LukE (32 kDa) and LukD (34.3 kDa) has been characterized from *Staphylococcus aureus* strain Newman. LukE was 58–68% identical with the class S proteins, whereas LukD was 71–77% identical with the class F proteins of the family. A partial immunoreactivity with the various affinity-purified antibodies specific for the other proteins was observed. Immunoprecipitation assay and gene probing confirmed a 30% frequency among human clinical isolates, differing from the distribution of the other known leucotoxins ($P < 0.005$). LukE+LukD was as effective as the Panton-Valentine leucocidin for inducing dermonecrosis when injected in the rabbit skin, but not hemolytic and poorly leucotoxic compared to other leucotoxins expressed by *Staphylococcus aureus*.

Key words: Characterization; Bi-component leucotoxin; LukE+LukD; Ca^{2+}/ethidium penetration; Dermonecrosis; *Staphylococcus aureus*

1. Introduction

Staphylococcal bi-component leucotoxins are composed of two distinct proteins: S-related (Slow eluted: 31–32 kDa) or F-related proteins (Fast eluted: 36–38 kDa) that are secreted separately, but act synergistically upon human polymorphonuclear cells or erythrocytes (PMNs). They constitute a family of pore-forming toxins comprising the anciently described Panton-Valentine leucocidin (PVL = LukS-PV+LukF-PV) [29], and γ-hemolysin, self-comprising two leucotoxic entities HlgA+HlgB and HlgC+HlgB where HlgA and HlgC are S-related components and HlgB is an F-related component [6,22]. The previous leucocidin R and that encoded by a methicillin-resistant *S. aureus* are bi-component leucotoxins comparable to the latter [13,22,28]. Recently, another S-related protein, LukM, which is also coupled to a class F component, LukF-PV like (LukF′-PV in this text), was characterized [14]. Another leucotoxin, LukS-I+LukF-I was reported to be produced by *Staphylococcus intermedius* strains [23]. These components were shown to be predominantly constituted of β-sheet structures [20]. Genes encoding PVL are distributed in 2% of clinical strains, strongly associated with furuncles [7,21]. γ-Hemolysin encoding genes are encountered in 99% of clinical isolates of *S. aureus* [21]. Any combination of a given class S protein with a class F protein may be toxic [22] and generates an intense inflammatory response when injected in the rabbit skin [8] or in the pars plana of the rabbit eye [25].

Sequential binding of S components to cell membranes is a prerequisite for that of F components [5]. This provokes a Ca^{2+} influx by opening of Ca^{2+} channels [27], and the activation of target cells [15,16]. Then, the leucotoxin forms pores through the plasma membrane which are sensitive to both Na^+, K^+, or ethidium [10,19,27].

The aim of this work was to characterize a cross-immunoreactive product with PVL components, and to demonstrate its effective membership in this leucotoxin family.

2. Materials and methods

2.1. Bacteria and animals

Escherichia coli XL1 Blue (Stratagene) was used as a recipient strain of the reduced genomic library cloned into the shuttle plasmid (*E. coli*/*S. aureus*) pCU1 [2], kindly given by F. Götz (Tübingen, Germany). *S. aureus* Newman (NTCC 8178) and P83 strains were used for the purification of LukE/LukD and LukM/LukF′-PV [14], respectively. For the toxin-production study, 127 consecutive clinical strains from 127 individuals were collected from the routine laboratory of the Strasbourg University Hospital.

2.2. Protein purification

One colony of Newman strain was grown for 8 h in 5 ml of YCP (3% (w/v) yeast extract (Oxoid), 2% (w/v) bacto-casaminoacids (Difco), 2% (w/v) sodium pyruvate (Merck), 0.25% (w/v) Na_2HPO_4, 0.042% (w/v) KH_2PO_4, pH 7.0) at 37°C. Then, the purification procedure was as described earlier [9,27], involving MonoS cation-exchange FPLC (Fast Performance Liquid Chromatography, Pharmacia, Uppsala, Sweden) and AlkylSuperose hydrophobic FPLC, respectively. Purified compounds were controlled by SDS-PAGE electrophoresis on PHAST System (Pharmacia) and then stored at −80°C at $OD_{280nm} = 1.0$ in 50 mM Na-phosphate buffer, 150 mM NaCl, pH 7.0.

2.3. Nucleic acids methods

DNA modifying and restriction enzymes were used as recommended by the manufacturers (New England Biolabs-Gibco-BRL). A synthetic 5′ ^{32}P-labelled oligonucleotide 5′-GAAAATATA/TGGA/TGATGGA/TGCA/TGAAGTTAT-3′ whose sequence was deduced from the N-terminal sequence of LukE: NTNIENIGDGAEVIKRTE was used as a probe for cloning *lukE* and *lukD*. A total DNA [28] library of Newman strain reduced to *Hin*dIII DNA fragments ranging from 2.0 to 3.0 kb inserted into pCU1 was screened as previously described [23]. Universal and reverse primers and the cloning oligonucleotide were initially used for nucleotide sequence determination of double stranded DNA with T7 DNA polymerase [24]. Other primers were deduced from the previously read sequences. Total RNA from 1 ml of 10-, 13-, and 16-h cultures of Newman strain was prepared by the Fast Prep (Bio101) method [4]. Southern and Northern blots [24] were performed by using QIABRANE membrane (Qiagen, Paris). For the determination of the transcription start of *lukE-lukD*, total RNA of Newman strain was submitted to extension with the 5′ ^{32}P-labelled oligonucleotide 5′-552-TCCTACTGCAAACTTGCAGCTAACA-3′-527. The reaction was carried out with 5 μg of RNA with a 1-h hybridization step at 65°C and a 45-min primer extension with an avian myeloblastosis virus reverse transcriptase (Appligene) as previously described [18]. DNA and peptide analyses were performed with DNAStar software (DNAStar, London, UK). The nucleotide sequence of *lukE*/*lukD* is recorded at the EMBL/GenBank library with the accession number Y13225.

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PII: S0014-5793(98)01130-2

2.4. Anti-LukE, -LukD, and -LukM antibodies and radial gel immunoprecipitations

Anti-LukS-PV and anti-LukF-PV affinity-purified rabbit polyclonal antibodies [9] were used for immunoblotting [24] during the purification of LukE and LukD. The rabbit polyclonal antibodies anti-LukE, anti-LukD and anti-LukM were affinity-purified [9]. All available antibodies were used in immunoprecipitation assays [9] for the detection of the production of PVL, LukE+LukD, and LukM+LukF′-PV by clinical isolates. A fresh colony of each strain was incubated in a 24-well cell culture plate filled with 550 μl of YCP medium, for 16 h at 37°C under a 10% CO_2 humid atmosphere with 180 rpm rotary shaking. To enhance the detection, immunoprecipitation plates were capillary dried. Proteins were precipitated for 5 min in 5% (w/v) trichloroacetic acid, and stained with 0.1% (w/v) Coomassie blue for 15 min, then washed five times with a 10% (v/v) acetic acid/30% (v/v) methanol solution, and fixed with a 10% (v/v) acetic acid/5% (v/v) glycerol solution. The χ^2 test was used to compare the different distributions of the leucotoxin-producing strains.

2.5. Preparation of human polymorphonuclear cells (PMNs)

Twelve ml of J-Prep solution (TechGen) were added to 30 ml of buffy coats from healthy donors and human PMNs were prepared as previously described [27]. Human PMNs were suspended and adjusted to 2×10^6 cells/ml with 140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.1 mM ethylene glycol-bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid, 10 mM HEPES, 3 mM Tris-base (pH 7.3). The latter buffer was used to wash, after loading the cells with the Ca^{2+}-specific fluorescent probe Fluo-3 (Molecular Probes, Eugene, OR, USA).

2.6. Flow cytometry measurements

Assays were made with 2×10^6 cells/ml loaded with 5 μM Fluo-3 during 1 h at 37°C, then washed and resuspended in the absence or presence of 1 mM free Ca_{2+}, and 25 nM ethidium bromide. Proteins were added at 2 nM. Flow cytometry measurements were performed by using a FacSort (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 5000 leucocytes (2×10^6 cells/ml) were classically gated [19]. Fluo-3 fluorescence due to the calcium penetration through opening of Ca^{2+} channels was recorded from the fluorescence light 1 ($\lambda_{Em}=530$ nm). The fluorescence light 3 ($\lambda_{Em}=650$ nm) was used to record the fluorescence of ethidium combined with nucleic acids, after penetrating through the leucotoxin pores. Data were calculated by Lysis 2TM software (Becton-Dickinson, France) and results expressed as mean ± S.D. of data obtained from PMNs of four different donors, at least.

2.7. Activity of LukE, LukD in a rabbit skin model

Shaved New Zealand rabbits (≥2.5) were injected intradermally with 50 μl of sterile 9.55‰ (w/v) PBS Dulbecco solution with equal amounts (100, 300, 1000, 3000, 10 000 ng) of class S- and class F-related components [8,22]. Clinical observations were recorded 12, 24, and 48 h after injection of toxins. Five reaction levels reflecting the intensity of the lesions were defined according to the evolution of the inflammatory response: level 0 was the normal skin, level 1 showed a red inflammatory reaction (∅ = 10–15 mm) without abscess formation, level 2 showed a hot, red papule with relief (∅ = 15–20 mm, 5 mm high), level 3 showed a more intense, blue and oedematous abscess (∅ = 20–30 mm, 10 mm high), level 4 still progressed (∅ = 30 mm, 10–15 mm high) with pus collection surrounded by blue oedema, level 5 showed a lesion with a central depression and pus sweating. Data obtained from three independent series of injections were analyzed with the non-parametric test of Wilcoxon-Mann-Whitney.

3. Results

3.1. New leucotoxin characterization

Characterization of a new leucotoxin from *S. aureus* strain Newman originated by the observation in culture supernates (Fig. 1A) of materials immunologically related to HlgC, LukS-PV, LukF-PV, but not with HlgA, HlgB and LukM, when the strain was grown in YCP medium. This observation evidenced that the supernatants contained proteins related to class S and class F components, which differed from PVL, γ-hemolysin or LukM/LukF′-PV. However, the Newman strain grown in HI medium then produced detectable amounts of HlgA, HlgB, and HlgC (Fig. 1A). By using the radial gel immunoprecipitation test, the class S protein arbitrarily called LukE and the class F protein called LukD were purified by using previously described chromatographies. The class S-related LukE protein was eluted on MonoS at a NaCl concentration of 280–290 mM similar to that of LukM. However, LukE was eluted in 1 M $(NH_4)_2SO_4$ by hydrophobic chromatography whereas LukM was not retained. LukD and LukF-PV were eluted at 110 mM NaCl onto MonoS and at 780 mM $(NH_4)_2SO_4$ onto alkyl-Superose. LukD and LukF-PV were produced by different strains. SDS-PAGE analysis of purified LukE and LukD confirmed apparent molecular masses similar to the other class S proteins (32 kDa) and to the other class F proteins (36 kDa), respectively (data not shown).

Affinity-purified antibodies were specific enough to distinguish amongst the related antigens (Fig. 1B), by generating continuous immunoprecipitation lines only with corresponding proteins and crossed immunoprecipitation lines between heterologous proteins. Few proteins were not precipitated by antibodies like LukE, LukS-PV and LukM by anti-HlgA antibodies, HlgC by anti-LukE antibodies, HlgA and LukM by anti-HlgC antibodies, or HlgA and LukS-PV by anti-LukM antibodies (Fig. 1B). Antigenic similarity seemed closer with LukD, HlgB, LukF-PV, LukF′-PV; LukF′-PV was not precipitated by anti-HlgB antibodies.

3.2. Cloning and sequencing lukE and lukD

Hybridization of the total DNA from Newman strain with the new leucotoxin-directed oligonucleotide probe (data not shown) revealed *Dra*I (2.1 kb), *Eco*RI (9 kb), *Eco*RV (>10 kb), *Hin*cII (1.9 kb), *Hin*dIII (2.8 kb), *Pst*I (>10 kb), *Sca*I (2.1 kb) DNA fragments differing in length from the *Eco*RI (>8.3 kb), *Eco*RV (4.2 kb), *Sca*I (3.8 kb) DNA fragments specific of the *hlg* locus, which seemed to be highly conserved in a majority of *S. aureus* isolates [21,22].

A clone containing a 2.8-kb *Hin*dIII insert was obtained and immunoblotting of the crude extract of the recombinant *E. coli* strain indicated the production of both LukE and LukD with apparent molecular masses comparable to those of the purified components (data not shown). Sequence analysis of a 2571-bp long nucleotide sequence overlapped two consecutive ORFs (Fig. 2) which spanned positions 511–1455 and 1457–2440, respectively. No open reading frame was found in the 500-bp preceding *lukE* differing from the γ-hemolysin locus. The two ORFs are separated by one thymidine as in other related genes (Fig. 2), and cotranscribed in a single mRNA expressed at the late exponential growth (Fig. 3A). Predictable −35 and −10 transcription signals and a putative ribosome binding site are reported in Fig. 2. The determination of the transcription start by the primer extension method (Fig. 3B) indicated the nucleotide 477, located 10 bases downstream the −35 box. *lukE* encodes a 314-amino acid protein with a calculated molecular mass of 35 222 Da and a calculated p*I* of 9.87. The sequence encoding a peptide NTNIEN..., located 28 amino acids dowstream the ATG initiation codon, corresponds exactly to the N-terminal sequence of LukE. This 28-residues long peptide bearing positively-charged residues followed by hydrophobic amino acids corresponds to the secretion signal peptide. The secreted 286-amino

acid LukE has a calculated molecular mass of 32 239 Da and a calculated p*I* of 9.88. Amino acid content of the secreted LukE revealed twice more basic than acidic residues (42:23). LukE had from 58.7% to 68% identity with the other class S components [6,14,22], with no conserved peptide sequence longer than 7 residues.

The second ORF, *lukD*, encodes a 327-amino acid-long protein with a calculated molecular mass of 36 974 Da and a calculated p*I* of 9.09. This nucleotide sequence is preceded by an rbs-like sequence. An inverted repeat probably involved in the termination of the transcription is located 60 bp downstream the stop codon of *lukD*. The 18 first sequenced N-terminal residues of the secreted LukD (AQNIT...), correspond to those deduced from the nucleotide sequence (position 1535). Therefore, the signal peptide of LukD, bears similar characteristics as above, contains 26 residues, and the secreted LukD is 301 amino acids long for a calculated molecular mass of 34 302 Da with a calculated p*I* of 9.02. The peptide sequence identity of LukD with class F components was comprised between 71 and 77% according to the proteins. Four domains were conserved between LukD and other class F components (from residues 34 to 52, 94 to 103, 137 to 150, 166 to 183). Despite a lack of sequence identity with α-toxin [12], homology was observed with both class S and class F proteins, confirming the observations described recently [11].

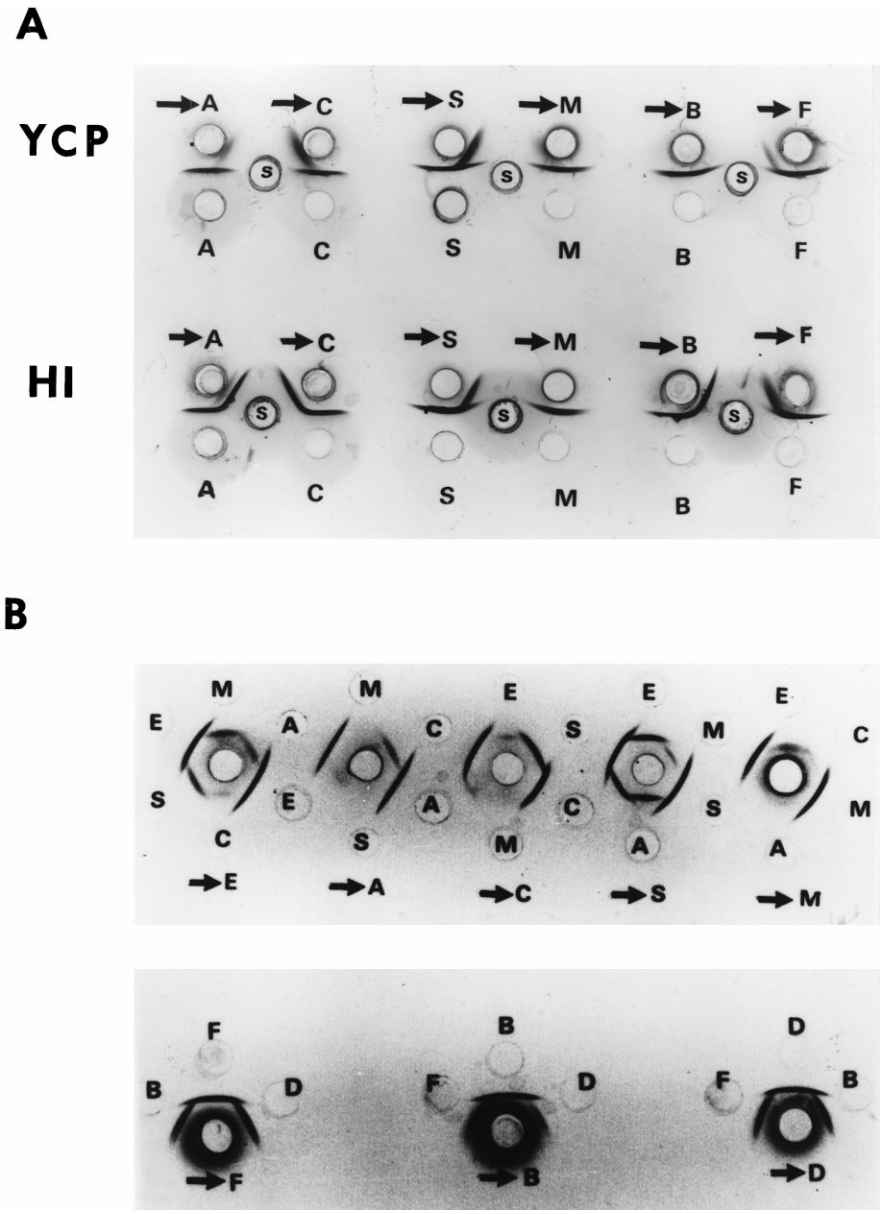

Fig. 1. Characterization of the new staphylococcal leucotoxin LukE/LukD. Panel A: Identification of materials immunologically related to bicomponent leucotoxins secreted by *S. aureus* Newman strain. Immunoprecipitation tests of culture supernatants (s) in YCP and HI media against 100 μg of affinity-purified rabbit antibodies anti-HlgA (→A), anti-HlgB (→B), anti-HlgC (→C), anti-LukF-PV (→F), anti-LukM (→M), anti-LukS-PV (→S) affinity purified rabbit polyclonal antibodies and 1 μg of HlgA (A), HlgB (B), HlgC (C), LukF-PV (F), LukM (M), LukS-PV (S). Panel B: Analysis by an immunoprecipitation assay stained with Coomassie blue of the antigenic relations between 1 μg of purified proteins constituting leucotoxins (HlgA: A; HlgB: B; HlgC: C; LukD: D; LukE: E; LukF-PV: F; LukM: M; LukS-PV: S), and 100 μg of the corresponding affinity-purified rabbit antibodies (→A, →B,...).

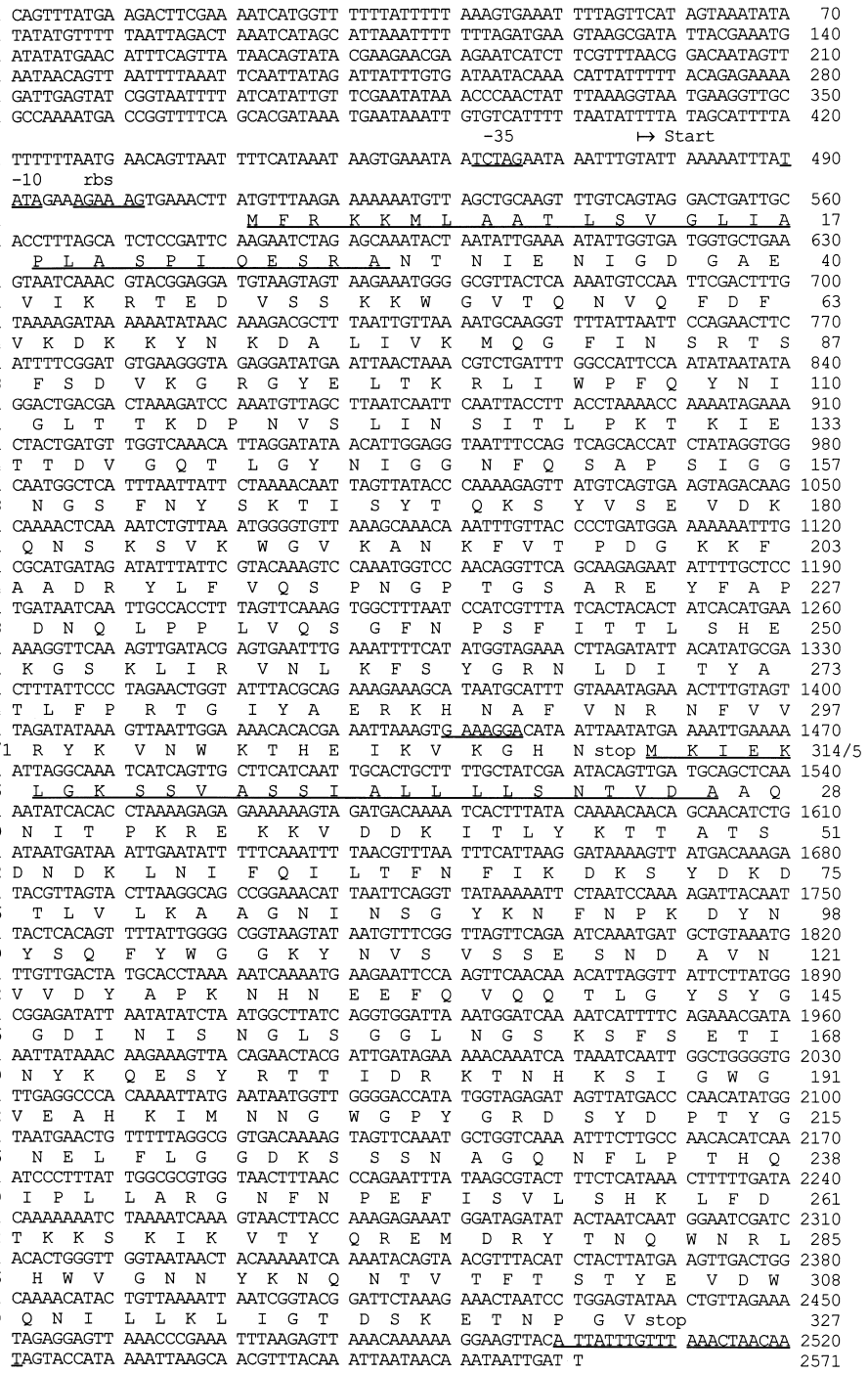

Fig. 2. Nucleotide sequence (EMBL/GenBank Y13225) of *lukE* (positions 511–1455) followed by that of *lukD* (positions 1457–2440) and the deduced peptide sequences from *S. aureus* Newman strain. The stop codon of *lukE* is located 1 base upstream the ATG codon of *lukD*. Signal peptides of the two proteins LukE and LukD are underlined. Putative consensus sequences (i.e. ribosome binding site (rbs) and promotor sequences −35 and −10) and the start point of the messenger RNA are mentioned. An inverted repeat sequence probably used as a transcription terminator (positions 2500–2521) is indicated by arrows.

3.3. *LukE+LukD and LukM production in 127 S. aureus clinical isolates*

For the study of the production of LukE+LukD among clinical isolates, attention was given to obtain significant series of isolates for each kind of sample. The immunoprecipitation test revealed no isolate producing LukM. Only one produced the PVL, which is in agreement with the previously described results [7,21]. However, 40 isolates (31.5%) produced LukE+LukD. This was confirmed by hybridization of *Hin*dIII-restricted DNAs with the *lukE*-oligonucleotide probe used for the mRNA mapping (data not shown). The hybridized DNA fragments were 3.1 and >8.3 kb long, according to the strains. No positive isolate was found among urine-originated samples, whereas a similar distribution concerned respiratory (6:29), skin samples (9:31), infected materials (8:19) and blood cultures (4:19). Most of the LukE+LukD-producing

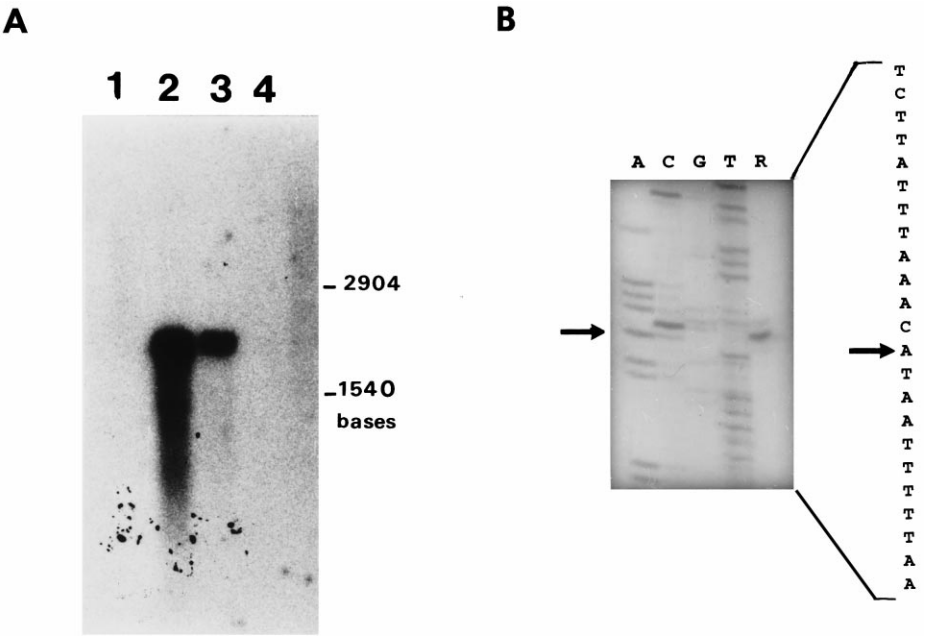

Fig. 3. Cotranscription of *lukE* and *lukD*, and determination of the corresponding mRNA start. A: Northern blot analysis of the total RNAs from *S. aureus* ATCC 49775 and Newman strains (YCP medium) with the labelled *lukE/lukD* genes. Lane 1: Total RNA from 10^8 CFU of ATCC 49775 after 12 h of culture. Lanes 2, 3, 4: Total RNA from 10^8 CFU of Newman after 12, 9, 6 h of culture, respectively. Lengths of the 16 s (1540 bases) and the 23 s (2904 bases) RNAs from *S. aureus* were obtained from formaldehyde-agarose gel stained with ethidium bromide. B: Primer extension indicated nucleotide A477 (T on the coding strand) as the mRNA start of *lukE/lukD*. Lanes A, C, G, T: Sequencing reactions; lane R, primer extension.

strains were isolated as pure cultures from diarrhea (77%) and were methicillin-resistant strains.

3.4. Biological activity

Such strains as Newman produce two leucotoxins among γ-hemolysin, PVL, and LukE+LukD. Class S-class F protein combinations were tested in hemolytic assays on rabbit (RRBC) and human (HRBC) red blood cells [22], on human PMNs and in a dermonecrosis assay in rabbit skin. None of the leucotoxin proteins alone induces any biological activity which excluded that they were contaminated. Neither LukE when combined with one of the class S components (i.e. LukS-PV, HlgA, HlgC, LukM), nor LukD combined with one of the class F components (i.e. LukF-PV, HlgB, LukF′-PV) harbored any biological activity.

3.4.1. In vitro assays. On HRBC, only the specific activities of LukE+HlgB = $7\times10^2 \pm 0.5\times10^2$, HlgA+LukD = $10^3 \pm 0.5\times10^2$ and HlgA+LukF′-PV = $10^4 \pm 2\times10^3$ U/mg were quantifiable but largely smaller than that of HlgA+HlgB ($5\times10^7 \pm 2\times10^6$ U/mg). On RRBC, the specific activities of HlgA+LukD = $3\times10^5 \pm 10^4$, and HlgA+LukF′-PV = $3\times10^6 \pm 10^5$ were significant, whereas the other LukE-, LukD-, LukM-, LukF′-PV-derived combinations remained basal compared to other leucotoxins [22].

The influx of Ca^{2+} in human PMNs provoked by the primary opening of Ca^{2+} channels [27] consecutive to leucotoxin activity was tested in the presence of 1 mM free Ca^{2+} for the 20 class S/class F protein combinations. The most potent couple HlgA+HlgB was assumed at 1000 arbitrary units of Fluo3-fluorescence from loaded PMNs and was compared with fluorescence intensities obtained with other pairs (Table 1). Cells were not lysed and the remaining lymphocytes were not susceptible to them. According to the calculated S.D. values, two kinds of results were obtained: (i) those presenting an S.D. value lower than the mean of assays; and (ii) those with a higher S.D. The first kind of results corresponded to pairs which were active with all the tested donors. HlgA was active in combination with all the F components, but the HlgA+LukD pair was the only combination with LukD presenting an activity. HlgA+LukF′-PV and LukS-PV+LukF-PV were as potent as HlgA+HlgB. HlgC was as active with HlgB as with LukF′-PV but less active with LukF-PV and LukD.

Table 1
Biological activities of the 20 possible leucotoxin combinations in *S. aureus* on the opening of Ca^{2+} channels of human PMNS

Components	HlgB	LukD	LukF-PV	LukF′-PV
HlgA	1000	349 ± 138	664 ± 163	907 ± 108
HlgC	497 ± 288	130 ± 245	183 ± 177	493 ± 246
LukS-PV	504 ± 242	100 ± 186	972 ± 183	672 ± 207
LukM	167 ± 180	99 ± 166	276 ± 197	295 ± 198
LukE	310 ± 277	90 ± 167	136 ± 211	166 ± 221

Data were obtained from the analysis of 5000 purified cells, previously charged with Fluo-3. The most lytic couple HlgA+HlgB was referred as 1000 arbitrary fluorescence units. Data are the mean ± S.D. of 4 independent experiments.

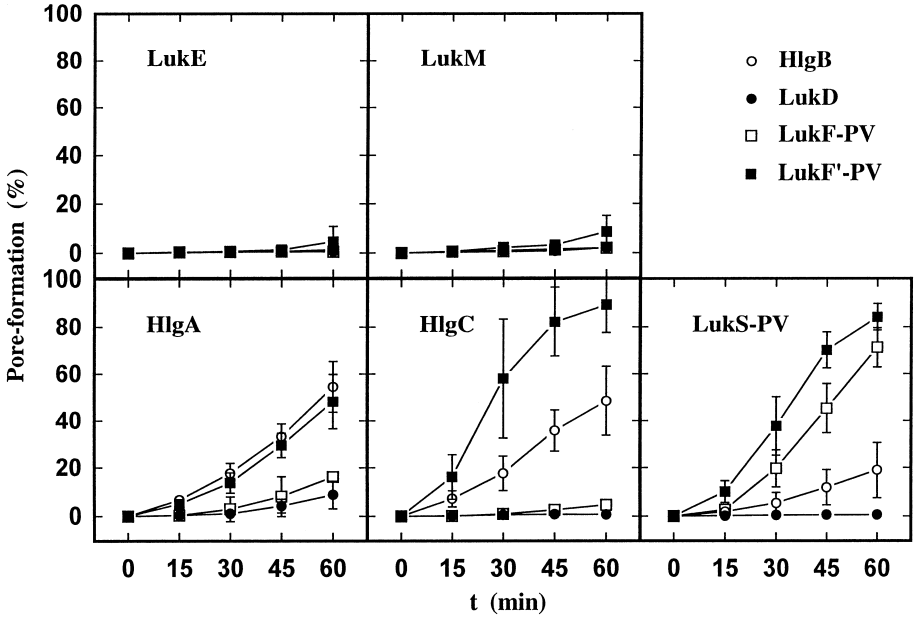

Fig. 4. The pore formation by the leucotoxins, in the absence of 1 mM Ca^{2+} out of human PMNs, was recorded by the entry of ethidium in 5000 purified cells, previously charged with Fluo-3. Data were obtained on FacSort (Becton-Dickinson), as the percentage of maximal cell lysis obtained after addition of 0.2% (w/v) of Triton X-100, and as the mean ± S.D. of 4 independent experiments. Twenty combinations of the mentioned class S components together with one class F component (○: HlgB, ●: LukD, □: LukF-PV, ■: LukF′-PV) are presented.

LukM presented the same activity with LukF′-PV or with LukF-PV. LukE+HlgB was representative of the second kind of results where leucotoxins appeared randomly active according to the donor. For a series of four donors, only one possessed PMNs more or less sensitive to these pairs. In the absence of Ca^{2+}, pore formation was studied by the entry of ethidium (Fig. 4). HlgA was the only S component presenting a little activity with LukD and all other class F components. LukF′-PV was the only class F component presenting activity with all class S proteins, although weak with LukE and LukM. LukF′-PV was the most potent F component with HlgC and with LukS-PV. The high S.D. value obtained for HlgC+LukF′-PV, particularly after a 30-min incubation, was due to variability of the lag-time preceding pore formation, according to the donor. In the presence of calcium, only few combinations as HlgA+HlgB or LukS-PV+LukF-PV formed pores and couples including LukE, LukM and LukD were devoid of activity (data not shown, [29]).

3.4.2. In vivo assays. Intradermal injections of leucotoxins in rabbit skin involving LukE, LukD and LukM, or LukF′-PV may induce a dermonecrotic reaction, according to the combinations. No variation was observed for rabbits receiving the same doses of leucotoxins ($P < 0.05$). Table 2 indicates that LukE+LukD was as potent as PVL, but the couples LukE+LukF-PV, LukM+LukD, LukE+LukF-PV, LukS-PV+LukD, HlgA+LukD, HlgC+LukD led to dermonecrotic lesions according to the doses and the delay after injections. Pairs containing HlgB, i.e. LukE+HlgB and LukM+HlgB were significantly less effective with no pus collection whatever the dose and the time following injections ($P < 0.05$). The

Table 2
Time course of dermonecrotic reactions after the intradermal injections of doses of functional leucotoxins including either LukE, or LukD or LukM proteins, and PVL as a reference [22]

Doses (ng)	100			300			1000			3000			10 000		
Time (h)	12	24	48	12	24	48	12	24	48	12	24	48	12	24	48
LukE+LukD	2	2	2	**3**	**4**	**4**	**3**	**5**	**5**	**4**	**5**	**5**	**4**	**5**	**5**
LukE+LukF-PV	1	1	2	2	3	3	**3**	**4**	**5**	**3**	**5**	**5**	**4**	**5**	**5**
LukE+HlgB	1	1	1	1	2	1	2	3	3	2	3	3	3	3	3
LukE+LukF′-PV	1	2	2	**2**	**4**	**4**	**3**	**4**	**5**	**3**	**5**	**5**	4	5	5
LukM+LukF′-PV	1	1	2	1	2	2	2	3	4	2	4	4	2	4	4
LukM+LukD	1	1	1	2	2	2	3	4	4	3	4	4	4	4	4
LukM+LukF-PV	1	1	1	1	2	3	2	3	4	2	4	4	3	4	5
LukM+HlgB	1	1	0	1	1	1	1	2	1	1	3	3	2	3	3
LukS+LukD	1	1	1	1	1	1	1	2	3	2	4	4	2	4	4
PVL	1	2	2	**3**	**4**	**4**	**3**	**4**	**5**	**4**	**5**	**5**	**4**	**5**	**5**
LukS-PV+LukF′-PV	1	2	2	2	3	3	2	4	4	3	4	5	4	4	5
HlgA+LukD	1	1	1	1	2	2	1	3	3	3	4	5	4	5	5
HlgA+LukF′-PV	1	1	1	1	1	2	2	2	3	2	3	4	2	3	4
HlgC+LukD	1	2	0	2	2	1	2	2	3	3	4	4	3	4	4
HlgC+LukF′-PV	1	1	1	1	1	2	2	2	3	2	3	3	2	3	4

Scores were attributed as described in Section 2.

reactions were always evolutive in the first 24 h, with slow healing from 5 days to 2 weeks, as previously reported [9,24].

4. Discussion

The new leucotoxin LukE+LukD characterized in this work remained undiscovered for two reasons. First, many of the LukE+LukD-producing *S. aureus* strains are able to produce simultaneously the γ-hemolysin whose components exhibit similar molecular masses and which cannot be distinguished by SDS-PAGE. The development of a defined medium like YCP medium was essential to determine the existence of LukE+LukD (Fig. 1A). Second, despite a marked dermonecrotic activity on rabbit skin, LukE+LukD is not strongly hemolytic or leucotoxic on human blood cells, as currently investigated for the other leucotoxins. HlgA+LukD was a Ca^{2+} channel inducer, whereas LukE+HlgB appeared only working according to the donors (Table 1). These observations suggest either different cell receptors or different ways in the processes of oligomerization for bi-component leucotoxins. Study of their binding sites should discard one of these hypotheses.

Partial antigenic communities between class S and class F proteins (Fig. 1B) and sequence identities of LukE/LukD with the other bi-component leucotoxins indicate that it belongs to this family of toxins. The 30% distribution of clinical strains of *S. aureus* producing LukE+LukD was significantly different ($P < 0.005$) from those of PVL- and γ-hemolysin producers [8,21].

Staphylococcal α-toxin and bi-component leucotoxins were suggested to possess similar structures allowing pore formation, especially for the rim and for the transmembrane stem (residues 100–150 of LukE, LukD) domains [11,26]. However, these toxins have functional differences. Class S components bind first to membrane ligands followed by secondary binding of class F components [5,19,20]. Privileged cell targeting is observed for PVL only active on cells derived from the metamyelocyte [10], whereas HlgA+HlgB and other leucotoxins [24] are more or less active on both PMNs and erythrocytes, or may permeate synthetic membranes [20]. α-Toxin is known to be active upon erythrocytes, lymphocytes, keratinocytes and fibroblasts, but rather not on PMNs [1,3]. Thr^{30} of HlgA and Thr^{28} of HlgC were important for the secondary binding of class F proteins [20]. The two cited residues and His^{35} from α-toxin also critical for its oligomerization [3,11,26], may be aligned. Therefore, oligomerization of these toxins may be mediated by differing residue interactions. Bi-component leucotoxins induce Ca^{2+}-channels [27], and form pores sensitive to monovalent cations. Other pore-forming toxins like α-toxin or aerolysin were not reported as calcium inducers [1,3,17]. Na^+, K^+, and ethidium were exchanged through the pores formed by leucotoxins [27], whereas those formed by α-toxin [3] and aerolysin [1] excluded ethidium and propidium.

Bi-component leucotoxins belong with α-toxin, aerolysin, protective antigen of the anthrax toxin [17] to a super-family of β-sheet structured pore-forming toxins. Those toxins previously named synergohymenotropic toxins [28] should be recalled bi-component leucotoxins because they differ from toxins as A/B bacteriocins and diphtheria toxin that acquire their biological activity by a synergy with membranes.

Acknowledgements: We thank F. Götz (Tübingen, Germany), T.J. Foster (Dublin, Ireland), Y. Kamio (Sendai, Japan), for kind gifts of the plasmid pCU1, the *S. aureus* Newman and P83 strains, respectively. Thanks are addressed to A. Staub from the IGBMC, Strasbourg, for protein sequencing. This work was supported by Grant EA1318 from the Direction de la Recherche et des Etudes Doctorales (DRED).

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