MAJOR ARTICLE

Immunogenicity of Toxins during *Staphylococcus aureus* Infection

Nelianne J. Verkaik,[1] Olivier Dauwalder,[3,4,5] Kenza Antri,[6] Ilhem Boubekri,[6] Corné P. de Vogel,[1] Cédric Badiou,[3,4] Michèle Bes,[3,4,5] François Vandenesch,[3,4,5] Mohamed Tazir,[6] Herbert Hooijkaas,[2] Henri A. Verbrugh,[1] Alex van Belkum,[1] Jerome Etienne,[3,4,5] Gerard Lina,[3,4,5] Nadjia Ramdani-Bouguessa,[6] and Willem J. B. van Wamel[1]

Departments of [1]Medical Microbiology and Infectious Diseases and [2]Immunology, Erasmus Medical Center, Rotterdam, the Netherlands; [3]Université Lyon 1, Centre National de Référence des Staphylocoques, [4]Institut National de la Santé et de la Recherche Médicale U851, and [5]Hospices Civils de Lyon, Lyon, France; and [6]Service de Microbiologie, Centre Hospitalo–Universitaire Mustapha Pacha, Algiers, Algeria

Background. Toxins are important *Staphylococcus aureus* virulence factors, but little is known about their immunogenicity during infection. Here, additional insight is generated.

Methods. Serum samples from 206 *S. aureus*–infected patients and 201 hospital-admitted control subjects were analyzed for immunoglobulin (Ig) G binding to 20 toxins, using flow-cytometry based technology. Antibody levels were associated with polymerase chain reaction–defined presence of toxin genes in homologous *S. aureus* isolates.

Results. IgG levels directed to exfoliative toxin (ET) A, ETB, γ hemolysin B (HlgB), leukocidin (Luk) D, LukE, LukS, staphylococcal enterotoxin (SE) A, SEE, SEH, SEI, and SElM were higher in *S. aureus*–infected patients than in control subjects ($P < .05$). Furthermore, in the *S. aureus*–infected patient group, IgG levels were higher if genes encoding ETA, ETB, SEA, SEC, SEH, SElQ, toxic shock syndrome toxin–1 (TSST-1), or Panton-Valentine leukocidin (PVL) were present in the infectious isolate ($P < .05$). Levels of anti-SEA IgG increased during infections with *sea*-positive (median fluorescence intensity from 11,555 to 12,388; $P < .05$) but not *sea*-negative strains. In addition, anti-LukS IgG levels increased during skin and soft-tissue infections with *luk-PV*–positive (median fluorescence intensity from 15,231 to 15,911; $P < .05$) but not *luk-PV*–negative strains. Bacteremia was associated with *sea* (odds ratio, 3.4; 95% confidence interval, 1.2–10.0) and *tst* (odds ratio, 5.7; 95% confidence interval, 1.6–20.8). Skin and soft-tissue infections and bone and joint infections were associated with *luk-PV* (odds ratio, 2.5; 95% confidence interval, 1.2–5.2).

Conclusions. Many toxins are expressed in vivo and recognized by the immune system during staphylococcal infections, suggesting their involvement in *S. aureus* pathogenesis.

Staphylococcus aureus produces numerous virulence factors that contribute to its ability to cause infections [1, 2]. These include a variety of toxins that are known for their detrimental effects on cells of the immune system [3]. In addition, their toxinogenic activity is implicated in a broad range of *S. aureus* infections [4]. Staphylococcal toxins can be categorized in groups: pyrogenic toxin superantigens (PTSAgs), exfoliative toxins (ETs), leukocidins, and other toxins. The family of PTSAgs includes staphylococcal enterotoxins (SEs), SE-like toxins [5], and toxic shock syndrome toxin-1 (TSST-1). Superantigens cross-link major histocompatibility complex class II molecules on antigen-presenting cells with T cell receptors, which leads to massive T cell proliferation and cytokine release [6]. This disproportionate proinflammatory activity is implicated in the pathogenesis of food poisoning and toxic shock syndrome [3, 7]. ETs are responsible for staphylococcal scalded skin syndrome and bullous impetigo [8]. Thus far, 4 ETs are known, and 3 of these (ETA, ETB, and ETD) are linked to human infection [9]. Leukocidal toxins constitute a family of pore-forming toxins that are composed of 2 distinct components. The toxic effect depends on the synergistic action of both class S and F proteins on human neutrophils or erythrocytes. Members of the leukotoxin family are LukD, LukE, LukM, γ hemolysin (Hlg), and Panton-Valentine leukocidin (PVL) [10, 11]. PVL is associated with

Received 11 May 2009; accepted 10 August 2009; electronically published 30 November 2009.

Reprints or correspondence: Dr Nelianne J. Verkaik, Dept of Medical Microbiology and Infectious Diseases, Erasmus MC, 's Gravendijkwal 230, 3015 CE Rotterdam, the Netherlands (n.j.verkaik@erasmusmc.nl).

Clinical Infectious Diseases 2010; 50:61–8

1058-4838/2010/5001-0010$15.00
DOI: 10.1086/648673

Table 1. Bacterial Strains and Sequences of Primers Used for *Staphylococcus aureus* Toxin Production

Toxin	*S. aureus* strains	*Escherichia. coli* strains and plasmids	Primers[a]	Restriction enzymes
SEA	A87 0502	M15 pQE30	CTC AGG ATC CAA TGG TAG CGA GAA AAG CG CTT TCT GCA GTT AAC TTG TAT ATA AAT ATA TAT CAA TAT GCA TG	BamHI/PstI
SEG	A99 0372	M15 pQE30	CAA TGG ATC CCC CGA TCT TAA ATT AGA CGA AC CGG ACT GCA GTC AGT GAG TAT TAA GAA ATA CTT CC	BamHI/PstI
SEI	A900322	M15 pQE30	CTA TGG ATC CGG TGA TAT TGG TGT AGG TAA C CGG ACT GCA GTT AGT TAC TAT CTA CAT ATG ATA TTT CGA C	BamHI/PstI
SEIM	A900322	M15 pQE30	GCA GGA TCC GAT GTC GGA GTT TTG AAT CTT AG CGG ACT GCA GTC AAC TTT CGT CCT TAT AAG ATA TTT C	BamHI/PstI
TSST-1	N315	BL21 pLys pIVEX 2.4d	TGG TAC TGG CGG CCGCTC TAC AAA CGA TAA TAT AAA GGA TTT G CGGACTGCAGT TAATTAATTT CTGCTTCTAT AGTTTTTATT TCATC	NotI/PstI
HlgB	ATCC 49775	BL21 pLys pIVEX 2.4d	TGG TAC TGG CGG CCG CGA AGG TAA AAT AAC ACC AGT C CGG GAT CCC TAT TTA TTG TTT TCA GTT TCT TTT GTA TC	NotI/BamHI
LukD	A87 0555	BL21 pLys pIVEX 2.4d	ACC CTT AAT TAA AGC TCA AAA TAT CAC ACC TAA AAG ACG CGG ATC CTT ATA CTC CAG GAT TAG TTT CTT TAG	PacI/BamHI
LukE	RN4220	BL21 pLys pIVEX 2.4d	ACG CGG ATC CTT AAT TAT GTC CTT TCA CTT TAA TTT ACC CTT AAT TAA AAA TAC TAA TAT TGA AAA TAT TGG TGA TGG TGC	BamHI/PacI

NOTE. HlgB, γ hemolysin B; Luk, leukocidin; SE, staphylococcal enterotoxin; SEI, staphylococcal enterotoxin-like; TSST-1, toxic shock syndrome toxin-1.

[a] Restriction sites are underlined.

necrotizing pneumonia, bone and joint infections, epidemic furunculosis, and abscesses in humans [12–15]. Toxins of the epidermal cell differentiation inhibitor family inactivate GTPases and thereby block important immune cell functions, such as chemotaxis and phagocytosis [16].

Despite the fact that toxins are important staphylococcal virulence factors and that the prevalence of *S. aureus* infection is continuously increasing [17], little is known about the immunoglobulin (Ig) G response directed against PTSAgs, ETs, and leukocidins during *S. aureus* infection in humans. By studying the immune response, important information can be collected concerning the antigenicity and in vivo expression of the toxins. This may increase knowledge on pathogenesis of *S. aureus* infection and might contribute to the development of new measures against staphylococcal disease. We studied the anti-toxin humoral immune response to 20 toxins in a large number of *S. aureus*–infected patients and hospital-admitted control subjects. Furthermore, we associated antibody levels with the presence of toxin genes in infectious *S. aureus* isolates, and we related toxin gene presence to different types of staphylococcal infection.

MATERIALS AND METHODS

Collection of serum and* S. aureus *strains. *S. aureus* isolates and 2 serum samples were collected from 206 *S. aureus*–infected patients in the Mustapha Pacha hospital (Algiers, Algeria) during 2006–2007. The first serum sample was obtained 5 days (range, 0–20 days) after strain identification. The second sample was collected 14 days (range, 7–34 days) thereafter. Serum samples were stored at −80°C until use. Data on sex, age, hospital ward, and type of infection were recorded. Isolates were considered to be community acquired if a sample obtained within 48 h after admission was culture positive for *S. aureus*. Isolates obtained later were considered to be hospital acquired. Furthermore, serum samples were collected for control patients ($n = 201$). Control patients were admitted in the same period but did not have an overt *S. aureus* infection. Patients with an immunocompromised status (eg, human immunodeficiency virus–positive patients or patients receiving corticosteroids or other immunosuppressive therapies) were excluded. Adult patients were defined as those aged ⩾18 years. All patients provided written informed consent, and the local Medical Ethics Committee of the Mustapha Pacha hospital approved the study.

Bacterial identification and toxin gene detection. *S. aureus* was identified on the basis of colony and microscopic morphology, coagulase testing with rabbit plasma (bioMérieux) and the Staphyslide agglutination test (bioMérieux). The identification was confirmed by multiplex polymerase chain reaction (PCR) amplification of the accessory gene regulator *(agr)* [18] and by determining the *agr* allelic group. The isolates were PCR-screened for genes encoding methicillin resistance (*mec*A); SE A, B, C, D, H, K, L, M, O, P, Q, and R *(sea-d, seh, selk-m,* and *selo-r);* TSST-1 *(tst);* ETA, ETB, and ETD *(eta, etb,* and *etd);* PVL *(luk-PV);* class F LukM leukocidin *(lukM);* HlgB *(hlgb);* and epidermal cell differentiation inhibitor *(edin),* as described elsewhere [18, 19].

Recombinant toxin production. The toxins SEC, SED, SEE, SEH, SElJ, SElN, SElQ, SER, ETA, ETB, LukF, and LukS were produced as described elsewhere [20–22]. The *S. aureus* strains listed in Table 1 were used to produce the other toxins. *Esch-*

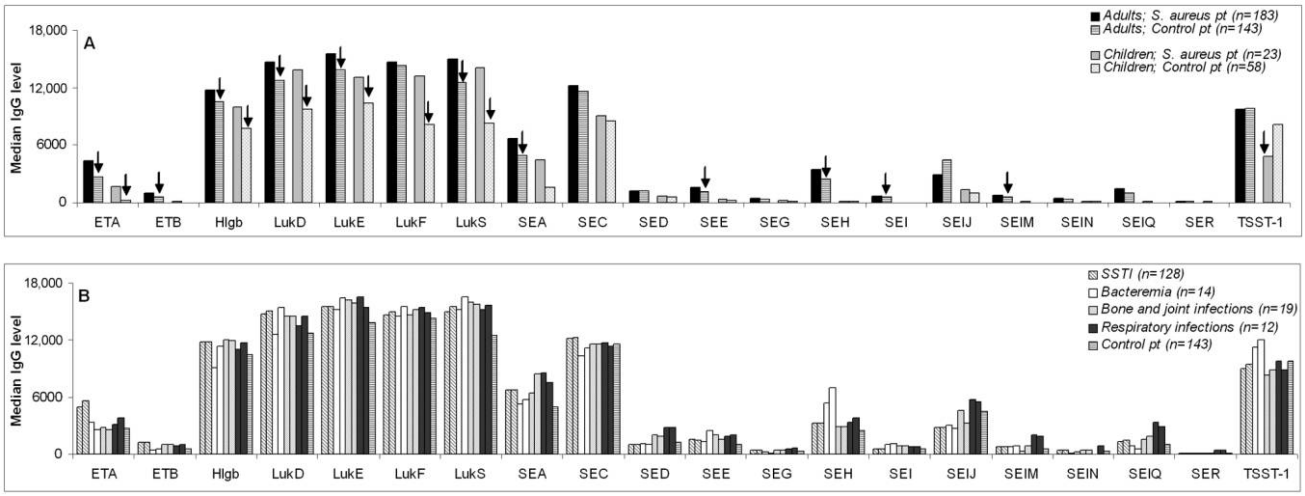

Figure 1. *A,* Toxin-specific immunoglobulin (Ig) G levels in *Staphylococcus aureus*–infected patients versus control patients and adults versus children. IgG levels are reflected by median fluorescence intensity (MFI) values. Black arrows indicate significant differences between the *S. aureus*–infected patient and control patient group ($P < .05$, by Mann Whitney *U* test). Results are shown for the first serum sample. *B,* Differences in toxin-specific IgG level between the first and second serum sample and between different types of *S. aureus* infections in adults. The first bar of each pattern represents median IgG level in the first serum sample; the second bar of the same pattern represents median IgG level in the second serum sample. ET, exfoliative toxin; HlgB, γ hemolysin B; Luk, leukocidin; SE, staphylococcal enterotoxin; SEl, staphylococcal enterotoxin-like; TSST-1, toxic shock syndrome toxin-1.

erichia coli M15 (Qiagen) and *E. coli* BL21 pLys (Invitrogen) were used for plasmid amplification and genetic manipulations. Primers were designed following the identification of suitable hybridization sites in the toxin genes (Table 1). Chromosomal DNA of *S. aureus* was extracted and used as a template for PCR amplification as described previously [23]. The 5′ primers were chosen within the coding sequence of each gene, omitting the region predicted to encode the signal peptide, as determined on the SignalP V3.0 World Wide Web Prediction Server (http://www.cbs.dtu.dk/services/SignalP/). The 3′ primers were chosen to overlap the stop codon of the toxin genes (Table 1). PCR products were codigested with appropriate restriction enzymes (Promega), purified with the High Pure PCR Product Purification kit (Roche) and ligated using T4 DNA Ligase (Roche) in either the pQE-30, pQE-70 (Qiagen), or pIVEX 2.4d expression vector (Roche) digested with the restriction enzymes described in Table 1 [24]. The resulting pQE plasmids were transformed into *E. coli* strain M15. For toxin expression in pIVEX 2.4d, the vector was transformed into *E. coli* strain DH5α (Invitrogen) before transformation into *E. coli* strain BL21 pLys. Open reading frame integrity was verified by sequencing the junctions between the plasmid and the insert.

Protein purification. Transformed *E. coli* cells growing exponentially in Luria-Bertani (LB) medium supplemented with ampicillin 100 μg/mL were inoculated into 1 liter of fresh LB medium and incubated with continuous rotary shaking for 2–3 h at 37°C until OD_{600} 0.5–0.7. Then, the expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM for 5 h. The cultures were then centrifuged at 4000 *g* for 20 min at 4°C. The cell pellets were stored overnight at −80°C. The cell pellets were thawed for 15 min on ice and resuspended in lysis buffer (Qiagen). The lysates were sonicated on ice after adding lysozyme. Then, the lysates were centrifuged at 10,000 *g* for 30 min at 4°C and the supernatants were collected. The His-tagged proteins were purified on Ni-nitrilotriacetic acid columns (Qiagen) and dialyzed against phosphate-buffered saline. Protein concentrations were determined according to the Bradford method, using bovine serum albumin as the standard. The toxins were quality controlled by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and mass spectrometry (Ultraflex MALDI-ToF; Bruker Daltonics).

Measurement of anti-toxin antibodies. Levels of IgG directed against the toxins ETA, ETB, Hlgb, LukD, LukE, LukF, LukS, SEA, SEC, SED, SEE, SEG, SEH, SEI, SElJ, SElM, SElN, SElQ, SER, and TSST-1 were quantified simultaneously using a bead-based flow cytometry technique (xMap; Luminex Corporation). Methods were as described before [25–27]. Tests were performed in independent duplicates, and the median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels, were averaged. In each experiment, control beads (no toxin coupled) were included to determine nonspecific binding. In the event of nonspecific antibody binding, the nonspecific MFI values were subtracted from the antigen-specific results. Human pooled serum was used as a standard [26].

Statistical analysis. Statistical analyses were performed with SPSS software, version 15.0 (SPSS). Kruskal-Wallis and Mann-Whitney *U* tests were used to compare differences in

Table 2. Presence of Toxin Genes in *Staphylococcus aureus* Strains Isolated from Patients with Different Types of *S. aureus* Infection

	No. (%) of gene-positive isolates							
Toxin gene	SSTI (n = 142)	Bacteremia (n = 18)	Bone infection (n = 22)	Respiratory infection (n = 12)	Other infection (n = 12)	Total (n = 206)	OR (95% CI)[a]	P
sea	20 (14)	**6 (33)**	1 (5)	2 (17)	1 (8)	30 (15)	3.4 (1.2–10.0)	.024
seb	6 (4)	0 (0)	2 (9)	0 (0)	0 (0)	8 (4)	…	NS
sec	4 (3)	1 (6)	1 (5)	0 (0)	0 (0)	6 (3)	…	NS
seh	9 (6)	2 (11)	0 (0)	0 (0)	0 (0)	11 (5)	…	NS
selk	11 (8)	2 (11)	1 (5)	1 (8)	1 (8)	16 (8)	…	NS
sell	4 (3)	1 (6)	1 (5)	0 (0)	0 (0)	6 (3)	…	NS
selm	32 (23)	6 (33)	7 (32)	3 (25)	5 (42)	53 (26)	…	NS
selo	32 (23)	6 (33)	7 (32)	3 (25)	5 (42)	53 (26)	…	NS
selp	6 (4)	0 (0)	1 (5)	0 (0)	0 (0)	7 (3)	…	NS
selq	11 (8)	2 (11)	1 (5)	1 (8)	1 (8)	16 (8)	…	NS
tst	6 (4)	**4 (22)**	0 (0)	1 (8)	2 (17)	13 (6)	5.7 (1.6–20.8)	.009
eta	2 (1)	0 (0)	0 (0)	1 (8)	0 (0)	3 (2)	…	NS
etb	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	…	NS
etd	70 (49)	6 (33)	9 (41)	4 (33)	5 (42)	94 (46)	…	NS
luk-PV	**71 (50)**	5 (28)	**11 (50)**	4 (33)	3 (25)	94 (46)	2.5 (1.2–5.2)	.015
hlgb	6 (4)	3 (17)	2 (9)	0 (0)	1 (8)	12 (6)	…	NS
edin	73 (51)	7 (39)	11 (50)	4 (33)	6 (50)	101 (49)	…	NS

NOTE. Boldfaced font indicates genes that are significantly more prevalent in strains isolated from a particular type of infection. CI, confidence interval; edin, epidermal cell differentiation inhibitor; et, exfoliative toxin; hlgb, γ hemolysin; luk-PV, Panton-Valentine leukocidin; NS, not significant; OR; odds ratio; se, staphylococcal enterotoxin; sel, staphylococcal enterotoxin-like; SSTI, skin and soft-tissue infection; tst, toxic shock syndrome toxin-1.

[a] ORs were calculated by use of binary logistic regression.

anti-toxin antibody levels between patients and control subjects. The χ^2 test was applied for categorical variables and binary logistic regression to calculate odds ratios (ORs). To compare antibody levels in the first and second serum sample of each patient, the Wilcoxon matched pairs signed rank test was used. $P < .05$ was considered statistically significant.

RESULTS

Patient characteristics. The group of 206 *S. aureus* infected patients consisted of 183 adults and 23 children. Their median age was 40 years (range, 0–84 years). Male-to-female ratio was 1.5:1. Of the 206 patients, 142 (69%) had skin and soft tissue infections (SSTIs; eg, furunculosis, infected skin lesions, or folliculitis); 18 (9%) had bacteremia; 22 (11%) had bone or joint infections (eg, arthritis or osteitis); 12 (6%) had respiratory infection (eg, bronchitis); 3 (1%) had ocular infection (eg, conjunctivitis or endophthalmitis); 2 (1%) had urinary tract infection; 4 (2%) had ear, nose, or throat infection (eg, sinusitis); and 3 (1%) had central nervous system infection. The group of 201 control patients consisted of 143 adults and 58 children. Median age was 45 years (range, 0–92 years) and the male-to-female ratio was 1.1:1.

Validation and inter-assay variation of the toxin multiplex assay. The MFI values obtained for human pooled serum with the multiplex assay (serum incubated with the differently fluorescence-colored antigen-coupled beads mixed in 1 well) were between 90% and 111% (median 96%) of the MFI values obtained with the individual assays (serum incubated with each individual color of antigen-coupled beads in separate wells). Therefore, the multiplex assay was considered reliable. Inter-assay variation was calculated from MFI values obtained for human pooled serum, which was included on each 96-wells plate. MFI values were averaged per protein. The median coefficient of variation was 11% (range, 5%–18%), which is comparable to what was found in other studies [25, 28]

Differences in anti-toxin IgG levels between distinct types of* S. aureus *infection. Antibody levels to 20 *S. aureus* toxins were measured. The toxin-specific antibody levels showed extensive inter-individual variability. IgG levels directed to 11 of 20 toxins (ETA, ETB, HlgB, LukD, LukE, LukS, SEA, SEE, SEH, SEI, and SElM) were significantly higher in adult *S. aureus*–infected patients than in adult control patients in both the first and second serum sample ($P < .05$) (Figure 1*A*). Adult patients with respiratory infections, as opposed to other *S. aureus* infections, had higher levels of IgG to SED (median MFI value, 2824 vs 1071; $P < .01$), SElJ (5510 vs 2815; $P < .01$), and SER (461 vs 133; $P < .05$) (Figure 1*B*). In adult patients with SSTI, the level of anti-ETA IgG was elevated (5657 vs 2848; $P < .05$).

Table 3. Difference in Presence of Toxin Genes between Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Methicillin-Susceptible *S. aureus* (MSSA) Isolates

	No. (%) of gene-positive isolates					
Toxin gene	MSSA (n = 112)	MRSA[a] (n = 94)	HA-MSSA (n = 66)	CA-MSSA (n = 46)	HA-MRSA (n = 61)	CA-MRSA (n = 33)
sea	28 (25)	2 (2)[b]	19 (29)	9 (20)	1 (2)	1 (3)
seb	8 (7)	0 (0)[b]	5 (8)	3 (7)	0 (0)	0 (0)
sec	6 (5)	0 (0)[c]	2 (3)	4 (9)	0 (0)	0 (0)
seh	11 (10)	0 (0)[b]	6 (9)	5 (11)	0 (0)	0 (0)
selk	10 (9)	6 (6)	6 (9)	4 (9)	5 (8)	1 (3)
sell	6 (5)	0 (0)[c]	2 (3)	4 (9)	0 (0)	0 (0)
selm	45 (40)	8 (9)[b]	23 (35)	22 (48)	7 (11)	1 (3)
selo	45 (40)	8 (9)[b]	23 (35)	22 (48)	7 (11)	1 (3)
selp	5 (5)	2 (2)	4 (6)	1 (2)	2 (3)	0 (0)
selq	10 (9)	6 (6)	6 (9)	4 (9)	5 (8)	1 (3)
tst	13 (12)	0 (0)[b]	9 (14)	4 (9)	0 (0)	0 (0)
eta	3 (3)	0 (0)	0 (0)	3 (7)	0 (0)	0 (0)
etb	1 (1)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
etd	16 (14)	78 (83)[b]	9 (14)	7 (15)	48 (79)	30 (91)
luk-PV	17 (15)	77 (82)[b]	7 (11)	10 (22)	47 (77)	30 (91)
edin	21 (19)	80 (85)[b]	13 (20)	8 (17)	50 (82)	30 (91)
hlgb	12 (11)	0 (0)[b]	8 (12)	4 (9)	0 (0)	0 (0)

NOTE. CA, community acquired; HA, hospital acquired.

[a] P values <.05 (by the χ^2 test) were considered to be statistically significant.

[b] $P < .01$.

[c] $P < .05$.

In the course of SSTI, respiratory and bone and joint infections, IgG levels showed no increase. In bacteremic patients, the level of IgG directed to HlgB, LukD, LukE, LukF, LukS, and SEE seemed to increase in the course of infection but this increase was not statistically significant ($P = .052$–$.084$) (Figure 1*B*).

In *S. aureus*–infected children, IgG levels directed to 6 of 20 proteins (ETA, HlgB, LukD, LukE, LukF, and LukS) were significantly higher ($P < .05$) (Figure 1*A*) than in children without *S. aureus* infections. Surprisingly, the level of anti-TSST-1 IgG was higher in young control patients (median MFI 4804 vs 8195; $P < .05$) than in *S. aureus*–infected patients. In the *S. aureus* patient group, IgG levels to 17 of 20 proteins were higher in adults than children ($P < .05$). No difference was shown in anti-LukD, anti-LukF, and anti-SER IgG. In the control group, IgG levels directed to all proteins were significantly higher in adults than children ($P < .001$) (Figure 1*A*).

Prevalence of genes encoding* S. aureus *toxins. The prevalence of genes encoding *S. aureus* toxins in the 206 infectious isolates is shown in Table 2. Of 206 isolates, 182 (88%) harbored ⩾1 toxin gene. The most prevalent genes were *edin, luk-PV,* and *etd,* which were detected in 49%, 46%, and 46% of isolates, respectively. Genes encoding SED, SER, and LukM were found in none of the isolates. Bacteremia was associated with a higher prevalence of the *sea* and *tst* gene. Isolates recovered from 6 (33%) of 18 bacteremic patients versus isolates from 24 (13%) of 188 other patients were *sea* positive. Isolates from 4 (22%) of 18 bacteremic patients versus isolates from 9 (5%) of 188 other patients were *tst* positive ($P < .05$ and $P < .01$, respectively) (Table 2). SSTI and bone and joint infections were associated with a higher prevalence of *luk-PV* ($P < .05$). Isolates recovered from 82 (50%) of 164 patients with SSTI or bone infection versus 12 (29%) of 42 other patients were *luk-PV* positive ($P < .05$) (Table 2).

Out of all infectious *S. aureus* isolates, 112 (54%) were methicillin susceptible (MSSA), and 94 (46%) were methicillin resistant (MRSA). The prevalence of *sea, seb, sec, seh, sell, selm, selo, tst,* and *hlgb* was higher among MSSA stains; *etd, luk-PV,* and *edin* were more prevalent among MRSA strains ($P < .05$) (Table 3). Within the group of MSSA or MRSA infections, the prevalence of toxin genes was not significantly different between hospital-acquired and community-acquired infections (Table 3).

Association of anti-toxin IgG levels with presence of toxin genes in infectious* S. aureus *isolates. Data on both gene presence and anti-staphylococcal antibody levels were available for 13 combinations (ETA, ETB, SEA, SEC, SED, SEH, SElM, SElQ, SER, TSST-1, HlgB, LukS, and LukF). There were no *sed*- and *ser*-positive isolates. Antibody levels were elevated if the gene was present in 8 *(eta, etb, sea, sec, seh, selq, tst,* and *luk-PV)* of 11 combinations ($P < .05$ (Figure 2). Furthermore, in patients with *sea*-positive *S. aureus* infection, the anti-SEA

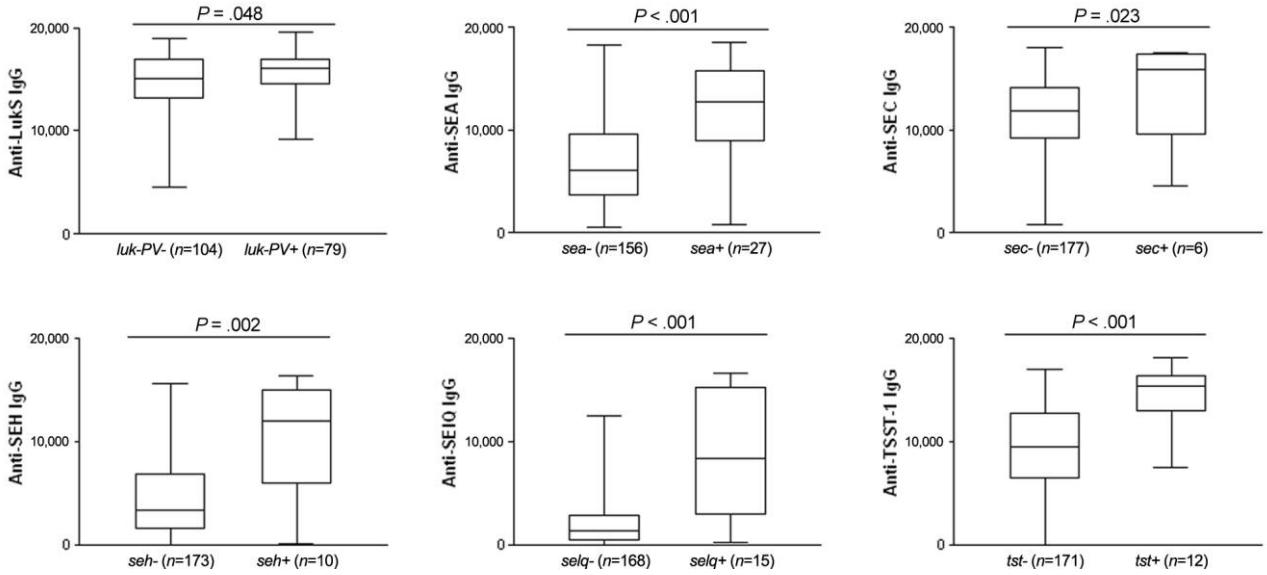

Figure 2. Box-and-whisker plots presenting the relation between toxin gene presences in isolates and the anti-toxin immunoglobulin (Ig) G levels in serum from *Staphylococcus aureus*–infected patients. Results for *eta* and *etb* are not shown because of the small number of gene positive isolates ($n = 3$ and $n = 1$, respectively). The box represents the 25th, 50th, and 75th percentile, the whisker represents the lowest and highest obtained value. Luk, leukocidin; SE, staphylococcal enterotoxin; SEI, staphylococcal enterotoxin-like; TSST-1, toxic shock syndrome toxin-1.

IgG titers increased significantly in the course of infection (median MFI, from 11,555 to 12,388; $P < .05$). In addition, in patients with a SSTI caused by a *luk-PV*–positive strain, the level of anti-LukS (not anti-LukF) IgG increased significantly (median MFI, from 15,231 to 15,911; $P < .05$). No increase in IgG level was seen in *sea*- and *luk-PV*–negative *S. aureus* infections.

DISCUSSION

For the majority of toxins, anti-staphylococcal IgG levels were higher in adults than children and IgG levels were higher in *S. aureus*–infected patients than in hospital-admitted control subjects (Figure 1*A* and 1*B*). This suggests that the anti-staphylococcal humoral immune state of an individual develops over the years and probably depends on the history of confrontations with *S. aureus*. IgG levels in serum of patients were higher if the toxin gene was present in their infectious isolates in 8 of 11 combinations (Figure 2). This indicates that *eta, etb, sea, sec, seh, selq, tst,* and *luk-PV* are actively expressed and stimulate the humoral immune system during *S. aureus* infections. For PVL and SEA, there was additional support for expression in vivo during *S. aureus* infections, as was found earlier by Croze et al [29] for PVL. The level of anti-SEA IgG increased in a time period of 2 weeks during infections caused by *sea*-positive but not *sea*-negative strains. The level of anti-SEE IgG did not increase, although earlier studies observed cross-reactivity with anti-SEA antibodies [36]. Additionally, the level of anti-LukS antibodies increased in the course of two weeks in patients suffering from SSTI caused by *luk-PV*–positive but not *luk-PV*–negative strains ($P < .05$). In contrast to LukS, IgG levels to LukF were not higher if isolates were *luk-PV* positive, and no increase anti-LukF IgG was detected in patients with a *luk-PV*–positive SSTI. In mice, a dominant anti-LukS IgG2a and 2b response developed after immunizing mice subcutaneously with both components. Furthermore, intranasally-vaccinated mice generated anti-LukS IgA, but not anti-LukF IgA [30]. Therefore, it seems that LukS is the dominant antigenic protein subunit, in mice as well as in humans.

For *S. aureus* infections that were caused by strains other than strains positive for *sea* or *luk-PV,* the level of toxin-specific IgG showed no increase in the course of infection. This suggests that, in the case of SSTI, respiratory and bone and joint infections, these infections did not elicit a strong systemic humoral immune response. Alternatively, differences in antibody levels between the 2 samples were nonsignificant because of the high premorbid anti-toxin IgG levels. High preexisting IgG levels might be the result of the high incidence of *S. aureus* infections in this particular population. Subsequently, this would suggest that these antibodies do not protect against these types of staphylococcal infection. Preformed anti-toxin IgG might also explain the high anti-SED and anti-SER IgG levels that were found in patients with a respiratory infection, even though the causative *S. aureus* isolates were *sed* and *ser* negative.

Bacteremia was associated with a high prevalence of *sea* and *tst,* SSTI and bone and joint infections with a high prevalence of *luk-PV,* in agreement with earlier studies [13, 31–33]. The most prevalent genes in the 206 clinical *S. aureus* isolates of

Algerian patients were *edin* (49%), *luk-PV* (46%), and *etd* (46%) (Table 2). These genes were more prevalent among MRSA than among MSSA strains ($P < .05$) (Table 3). Likely, this is due to the frequent occurrence of the *luk-PV*–, *etd*-, and *edin*-positive MRSA-ST80 clone that is predominant in Algeria [34, 35].

We measured IgG binding to toxins, but we do not have data on neutralizing capacity and cross-reactivity of these antibodies. In earlier studies, neutralizing capacity for anti-TSST-1, SEA, SEB, SEC, and SEE antibodies was observed [6]. Cross-reactivity was shown between anti-SEA and anti-SEE antibodies and between anti-SEB and anti-SEC antibodies [36, 37]. For other enterotoxins, antibody titers specific for heterologous toxins were 10-fold lower than those directed against the toxin used for immunization, which argues against a strong cross-reactivity [6, 38]. Knowledge on cross-reactivity and functionality of the anti-toxin antibodies should be increased, though. Furthermore, we do not know the *S. aureus* carrier state of the patients. Because nasal carriers of *S. aureus* have an increased risk of infection and nearly 80% of the infections are endogenous, their level of IgG might be influenced by colonization and/or previous infections with their colonizing strain [39, 40]. Therefore, including nasal swab cultures in future studies is important.

In conclusion, during *S. aureus* infection, the toxins ETA, ETB, SEA, SEC, SEH, SElQ, TSST-1, and Luk-PV are actively expressed and recognized by the humoral immune system. *S. aureus* bacteremia is associated with a high prevalence of *sea* and *tst* whereas SSTI and bone and joint infections are associated with presence of *luk-PV*. Significant increases in anti-SEA IgG and anti-Luk-PV IgG levels are observed during all types of infection and SSTI, respectively, suggesting their involvement in staphylococcal pathogenesis.

Acknowledgments

We thank Muriel Croze, Jean Philippe Rasigade, Caroline Bouveyron, Christine Courtier, Christine Gardon, Céline Spinelli, Annie Martra, Martine Rougier, Theo Hoogenboezem, Claudia Brandt-Hagens, and Diana Dufour-van den Goorbergh for their technical help.

Potential conflicts of interest. All authors: no conflicts.

References

1. Foster TJ, Hook M. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol **1998**; 6:484–8.
2. Chavakis T, Preissner KT, Herrmann M. The anti-inflammatory activities of *Staphylococcus aureus*. Trends Immunol **2007**; 28:408 –18.
3. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev **2000**; 13:16–34.
4. Lowy FD. *Staphylococcus aureus* infections. N Engl J Med **1998**; 339: 520–32.
5. Lina G, Bohach GA, Nair SP, Hiramatsu K, Jouvin-Marche E, Mariuzza R. Standard nomenclature for the superantigens expressed by Staphylococcus. J Infect Dis **2004**; 189:2334–6.
6. Holtfreter S, Roschack K, Eichler P, et al. *Staphylococcus aureus* carriers neutralize superantigens by antibodies specific for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis. J Infect Dis **2006**; 193:1275–8.
7. Stolz SJ, Davis JP, Vergeront JM, et al. Development of serum antibody to toxic shock toxin among individuals with toxic shock syndrome in Wisconsin. J Infect Dis **1985**; 151:883–9.
8. Amagai M, Matsuyoshi N, Wang ZH, Andl C, Stanley JR. Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. Nat Med **2000**; 6:1275–7.
9. Yamasaki O, Tristan A, Yamaguchi T, et al. Distribution of the exfoliative toxin D gene in clinical *Staphylococcus aureus* isolates in France. Clin Microbiol Infect **2006**; 12:585–8.
10. Gravet A, Colin DA, Keller D, Girardot R, Monteil H, Prevost G. Characterization of a novel structural member, LukE-LukD, of the bicomponent staphylococcal leucotoxins family. FEBS Lett **1998**; 436: 202–8.
11. Prevost G, Couppie P, Prevost P, et al. Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. J Med Microbiol **1995**; 42:237–45.
12. Gillet Y, Etienne J, Lina G, Vandenesch F. Association of necrotizing pneumonia with Panton-Valentine leukocidin-producing *Staphylococcus aureus*, regardless of methicillin resistance. Clin Infect Dis **2008**; 47:985–6.
13. Dohin B, Gillet Y, Kohler R, et al. Pediatric bone and joint infections caused by Panton-Valentine leukocidin-positive *Staphylococcus aureus*. Pediatr Infect Dis J **2007**; 26:1042–8.
14. Durupt F, Mayor L, Bes M, et al. Prevalence of *Staphylococcus aureus* toxins and nasal carriage in furuncles and impetigo. Br J Dermatol **2007**; 157:1161–7.
15. Issartel B, Tristan A, Lechevallier S, et al. Frequent carriage of Panton-Valentine leucocidin genes by *Staphylococcus aureus* isolates from surgically drained abscesses. J Clin Microbiol **2005**; 43:3203–7.
16. Czech A, Yamaguchi T, Bader L, et al. Prevalence of Rho-inactivating epidermal cell differentiation inhibitor toxins in clinical *Staphylococcus aureus* isolates. J Infect Dis **2001**; 184:785–8.
17. Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999–2005. Emerg Infect Dis **2007**; 13:1840–6.
18. Jarraud S, Mougel C, Thioulouse J, et al. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun **2002**; 70:631–41.
19. Tristan A, Ying L, Bes M, Etienne J, Vandenesch F, Lina G. Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. J Clin Microbiol **2003**; 41:4465–7.
20. Thomas D, Dauwalder O, Brun V, et al. *Staphylococcus aureus* superantigens elicit redundant and extensive human Vbeta patterns. Infect Immun **2009**; 77:2043–50.
21. Yamasaki O, Yamaguchi T, Sugai M, et al. Clinical manifestations of staphylococcal scalded-skin syndrome depend on serotypes of exfoliative toxins. J Clin Microbiol **2005**; 43:1890–3.
22. Prevost G, Cribier B, Couppie P, 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.
23. Jarraud S, Peyrat MA, Lim A, et al. egc, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J Immunol **2001**; 166:669–77.
24. Boisset S, Geissmann T, Huntzinger E, et al. *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. Genes Dev **2007**; 21:1353–66.
25. Verkaik N, Brouwer E, Hooijkaas H, van Belkum A, van Wamel W. Comparison of carboxylated and Penta-His microspheres for semi-quantitative measurement of antibody responses to His-tagged proteins. J Immunol Methods **2008**; 335:121–5.
26. Verkaik NJ, de Vogel CP, Boelens HA, et al. Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of *Staphylococcus aureus*. J Infect Dis **2009**; 199:625–632.

27. Martins TB, Augustine NH, Hill HR. Development of a multiplexed fluorescent immunoassay for the quantitation of antibody responses to group A streptococci. J Immunol Methods **2006**; 316:97–106.
28. Lal G, Balmer P, Stanford E, Martin S, Warrington R, Borrow R. Development and validation of a nonaplex assay for the simultaneous quantitation of antibodies to nine *Streptococcus pneumoniae* serotypes. J Immunol Methods **2005**; 296:135–47.
29. Croze M, Dauwalder O, Dumitrescu O, et al. Serum antibodies against Panton-Valentine leukocidin in a normal population and during *Staphylococcus aureus* infection. Clin Microbiol Infect **2009**; 15:144–8.
30. Brown EL, Dumitrescu O, Thomas D, et al. The Panton-Valentine leukocidin vaccine protects mice against lung and skin infections caused by *Staphylococcus aureus* USA300. Clin Microbiol Infect **2008**; 15:156–64.
31. Becker K, Friedrich AW, Lubritz G, Weilert M, Peters G, Von Eiff C. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. J Clin Microbiol **2003**; 41:1434–9.
32. Ferry T, Thomas D, Genestier AL, et al. Comparative prevalence of superantigen genes in *Staphylococcus aureus* isolates causing sepsis with and without septic shock. Clin Infect Dis **2005**; 41:771–7.
33. Peacock SJ, Moore CE, Justice A, et al. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect Immun **2002**; 70:4987–96.
34. Ramdani-Bouguessa N, Bes M, Meugnier H, et al. Detection of methicillin-resistant *Staphylococcus aureus* strains resistant to multiple antibiotics and carrying the Panton-Valentine leukocidin genes in an Algiers hospital. Antimicrob Agents Chemother **2006**; 50:1083–5.
35. Ben Nejma M, Mastouri M, Bel Hadj Jrad B, Nour M. Characterization of ST80 Panton-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* clone in Tunisia. Diagn Microbiol Infect Dis **2008** [Epub ahead of print].
36. Bergdoll MS, Borja CR, Robbins RN, Weiss KF. Identification of enterotoxin E. Infect Immun **1971**; 4:593–5.
37. Hynes WL, Weeks CR, Iandolo JJ, Ferretti JJ. Immunologic cross-reactivity of type A streptococcal exotoxin (erythrogenic toxin) and staphylococcal enterotoxins B and C1. Infect Immun **1987**; 55:837–8.
38. Bavari S, Ulrich RG, LeClaire RD. Cross-reactive antibodies prevent the lethal effects of *Staphylococcus aureus* superantigens. J Infect Dis **1999**; 180:1365–9.
39. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. N Engl J Med **2001**; 344:11–6.
40. Wertheim HF, Vos MC, Ott A, et al. Mupirocin prophylaxis against nosocomial *Staphylococcus aureus* infections in nonsurgical patients: a randomized study. Ann Intern Med **2004**; 140:419–25.