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ARTICLE

Heterogeneity of the humoral immune response following *Staphylococcus aureus* bacteremia

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Abstract Expanding knowledge on the humoral immune response in *Staphylococcus aureus*-infected patients is a mandatory step in the development of vaccines and immunotherapies. Here, we present novel insights into the antibody responses following *S. aureus* bacteremia. Fifteen bacteremic patients were followed extensively from diagnosis onwards (median 29 days, range 9–74). *S. aureus* strains (median 3, range 1–6) and serial serum samples (median 16, range 6–27) were collected. Strains were genotyped by pulsed-field gel electrophoresis (PFGE) and genes encoding 19 staphylococcal proteins were detected by polymerase chain reaction (PCR). The levels of IgG, IgA, and IgM directed to these proteins were determined using bead-based flow cytometry. All strains isolated from individual patients were PFGE-identical. The genes encoding clumping factor (Clf) A, ClfB, and iron-responsive surface-determinant (Isd) A were detected in all isolates. Antigen-specific IgG levels increased more frequently than IgA or IgM levels. In individual patients, different proteins induced an immune response and the dynamics clearly differed. Anti-ClfB, anti-IsdH, and anti-fibronectin-binding protein A IgG levels increased in 7 of 13 adult patients ($p<0.05$). The anti-IsdA IgG level increased in 12 patients (initial to peak level: 1.13–10.72 fold; $p<0.01$). Peak level was reached 7–37 days after diagnosis. In a bacteremic 5-day-old newborn, antistaphylococcal IgG levels declined from diagnosis onwards. In conclusion, each bacteremic patient develops a unique immune response directed to different staphylococcal proteins. Therefore, vaccines should be based on multiple components. IsdA is immunogenic and, therefore, produced in nearly all bacteremic patients. This suggests that IsdA might be a useful component of a multivalent staphylococcal vaccine.

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Introduction

Staphylococcus aureus is a leading cause of nosocomial bloodstream infections [1]. Risk factors for these invasive infections are intravascular catheters and nasal carriage [2]. Nasal carriers have a three- to four-fold increased risk of acquiring a nosocomial bacteremia as compared to non-carriers [3, 4]. Bacteremic patients can develop serious complications, such as infective endocarditis, prosthetic device infection, septic arthritis, deep tissue abscesses, and vertebral osteomyelitis [5–8]. *S. aureus* bloodstream infections extend the length of hospital stay and increase antibiotic usage, costs, and mortality; approximately 20–30% of the patients die [1, 9, 10]. Worldwide, the increasing resistance of *S. aureus* isolates to various antibiotics complicates the treatment of bacteremia [11, 12]. Meanwhile, the number of new approved antimicrobial agents has decreased over the last several years [13]. Therefore, alternative strategies to prevent and treat *S. aureus* bacteremia, such as vaccines and immunotherapy, are urgently required [14]. For the development of these alternative strategies, expanding knowledge on the humoral immune response in *S. aureus*-infected patients is a mandatory step. In this study, we generate novel insights in the antibody responses following *S. aureus* bacteremia.

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Materials and methods

Patients, definitions, and setting

Fifteen patients (13 adults and two children, Table 1) admitted to the Erasmus MC, Rotterdam, The Netherlands, from March to June 2008 were followed from the diagnosis of *S. aureus* bacteremia until the end of hospital stay (range 9–74 days, median 29 days). Bacteremia was defined upon isolation of *S. aureus* from at least one blood culture set. From these patients, a total of 44 methicillin-susceptible *S. aureus* strains were collected for routine culture. The median number of collected strains per patient was 3 (range 1–6). The *S. aureus* strains were isolated from blood, sites of infection, and, if applicable, other sites. Furthermore, serial serum samples were collected (leftover material). The median number of days between the first positive blood culture and the first sampling of serum was 1 day (range 0–20 days). From each patient, at least 6 (median 16, range 6–27) serum samples were collected, leading to a total of 232 serum samples. The time between the first positive blood culture and the first negative (control) blood culture ranged from 1–6 days (median 2 days; excluding one patient for whom no additional blood culture was obtained).

Sera from four non-*S. aureus* bacteremic patients were used as controls. These four patients were diagnosed with a *Klebsiella pneumoniae*, *Proteus mirabilis*, coagulase-negative staphylococcus (CNS), or *Enterococcus faecium* bacteremia. The number of days between the first positive blood culture and the first sample ranged from 0 to 2 days. Per patient, 12–26 (median 16) serum samples were collected. The follow-up time ranged from 22 to 50 days (median 29 days).

Patients were treated with antibiotics according to hospital guidelines under the supervision of Infectious Disease consultants. Catheters were removed if they were the suspected origin of the bacteremia. The Medical Ethics Committee of the Erasmus MC, Rotterdam, The Netherlands, approved the study (MEC 2007-106, addendum 2).

S. aureus identification, detection of virulence genes, and genotyping

S. aureus was identified based on colony and microscopic morphology and Slidex Staph Plus agglutination testing (bioMérieux). The identification of *S. aureus* was confirmed by *spa* polymerase chain reaction (PCR) [15]. The isolates were screened for genes encoding important staphylococcal proteins: the microbial surface components recognizing

Table 1 Characteristics of the patients and their bacteremia

Patient no.	Sex	Age (years)	Underlying disease	Origin of *S. aureus* bacteremia[a]	Outcome	No. of samples
1	F	21	Kidney transplantation	Catheter related	Survived	27
2	M	71	B-cell non-Hodgkin lymphoma	Catheter related	Survived	8
3	M	21	HIV positive	Pyomyositis	Survived	14
4	F	74	DM II, cortisol-producing tumor of the adrenal gland	Abscess hand (catheter related)	Survived	26
5	M	60	DM II and cardiovascular disease	Unknown	Deceased[b]	9
6	M	75	DM II and cardiovascular disease	Diabetic foot	Survived	19
7	M	63	Cardiovascular disease	Osteomyelitis sternum (after CABG)	Survived	23
8	M	69	Esophageal carcinoma	Cellulitis knee	Survived	7
9	M	37	Myocarditis	Infected thrombus (catheter related)	Survived	18
10	M	64	Bronchus carcinoma	Unknown	Survived	22
11	M	63	None	Spondylodiscitis (dental origin)	Survived	8
12	F	64	Mamma carcinoma T-cell lymphoma	Catheter related	Survived	16
13	F	47	IV drug abuse Hepatitis C	Chronic osteomyelitis arm	Deceased (MOF by sepsis)	9
14	M	5 days	Congenital disorders	Infected head wound (catheter related)	Survived	20
15	M	2 years 11 months	Medulloblastoma	Catheter related	Deceased[b]	6

M, male; F, female; DM, diabetes mellitus; CABG, coronary artery bypass graft; MOF, multiple organ failure

[a] The origin of the bacteremia was recorded on the medical charts by the Infectious Disease consultant

[b] Cause of death not related to bacteremia according to post-mortem examination

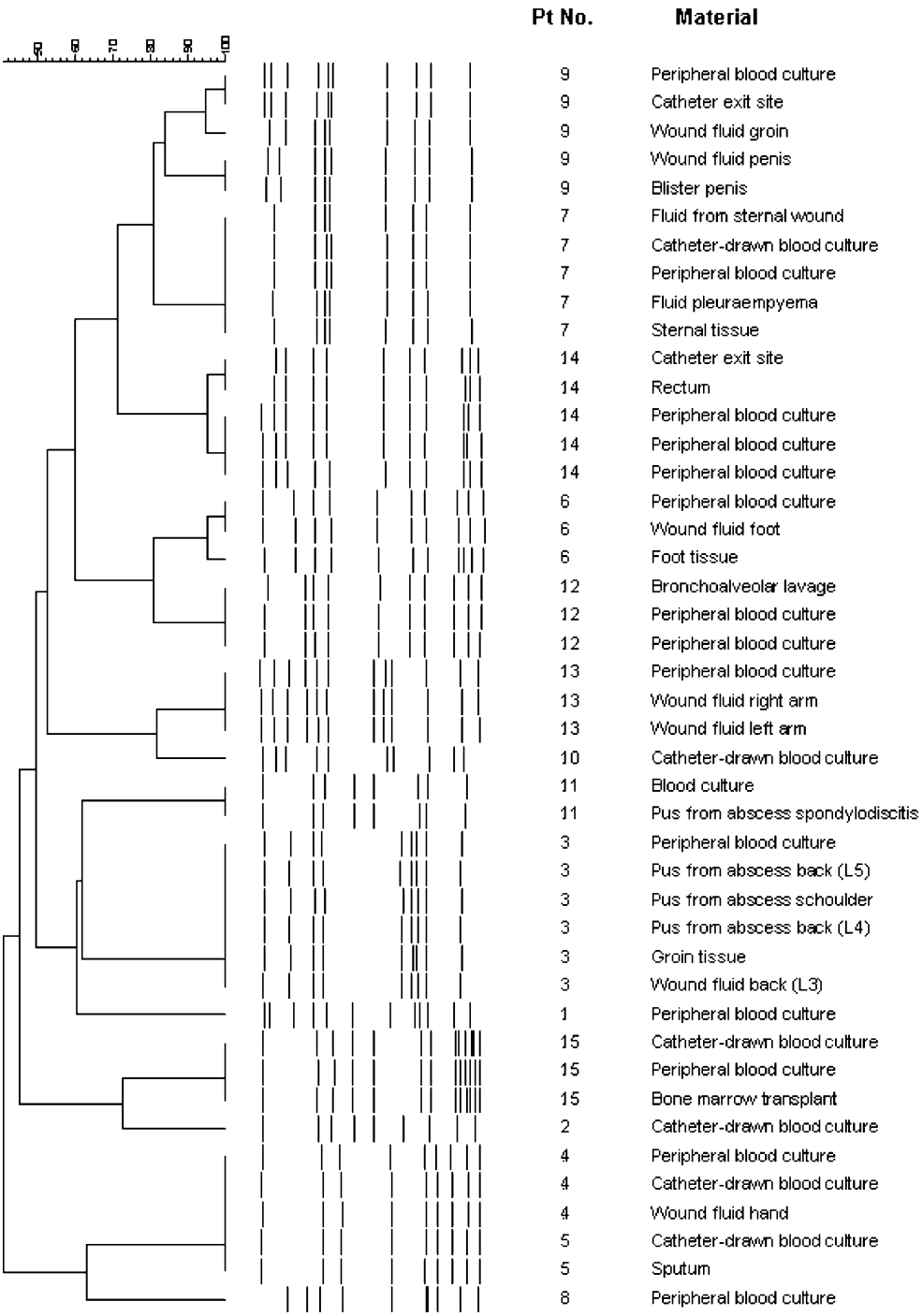

Fig. 1 Dendogram of the pulsed-field gel electrophoresis (PFGE) data of 44 *Staphylococcus aureus* strains isolated from 15 bacteremic patients. Strains isolated from individual patients are clonally related

adhesive matrix molecules (MSCRAMMs), staphylococcal enterotoxins (SEs), and immunomodulatory proteins [16–20]. Sequences specific for clumping factor A and B (ClfA and ClfB), *S. aureus* surface protein G (SasG), iron-responsive surface determinant A and H (IsdA and IsdH), fibronectin-binding protein A and B (FnbpA and FnbpB), serine-aspartate dipeptide repeat protein D and E (SdrD and SdrE), SEA, SEB, SEI, SEM, SEO, SEQ, toxic shock syndrome toxin (TSST-1), staphylococcal complement inhibitor (SCIN), extracellular fibrinogen-binding protein (Efb), and chemotaxis inhibitory protein of *S. aureus* (CHIPS) were detected. Primers for *isdA* (Fw, CTGCGTCAGCTAATGTAGGA; Rv, TGGCTCTTCAGAGAAGTCAC), *isdH* (Fw, TCTGCTGGTGGATACTGTTG; Rv, TGCCAGTGAGACTTGTATCG), *sasG* (Fw, GGCCACTTGGATGAGTTGGT; Rv, CGAAGAGCCAGTGGATGATG), *sdrD* (Fw, CGGAGCTGGTCAAGAAGTAT; Rv, TGCCATCTGCGTCTGTTGTA), and *efb* (Fw, GAAGGATACGGTCCAAGAGA; Rv, TGTGGACGTGCACCATATTC) were newly designed. Other genes were detected by PCR as described

Table 2 Fold increase from initial antigen-specific antibody level to peak antibody level in 13 adult patients suffering from an *Staphylococcus aureus* bacteremia

Patient no.		CHIPS	SCIN	Efb	ClfA	ClfB	FnbpA	FnbpB	IsdA	IsdH	SasG	SdrD	SdrE	SEA	SEB	SEI	SEM	SEO	SEQ	TSST-1
1	IgG	4.6 +	2.1 +	1.8 +	2.3 +	1.5 +	1.9 +	−	2.3 +	2.1 +	−	19.4 +	+	−	−	+	+	+	−	−
	IgA	1.6		1.4		1.7														
	IgM	2.6	3.1		1.6		10.4	*16.4*	1.8		*6.8*		10.9							
2	IgG	+	+	−	1.6 +	1.2 +	2.0 +	−	2.5 +	−	−	5.8 +	2.0 +	−	−	1.3 +	1.3 +	+	−	*1.3* −
	IgA	1.2							2.8				2.0			2.2	2.0			
	IgM	1.6	1.4						2.6				3.4		*1.2*	3.7	1.7			*1.3*
3	IgG	+	+	1.5 +	1.5 +	1.5 +	1.4 +	+	1.2 +	+	+	+	+	2.4 +	−	−	−	−	−	−
	IgA		1.1																	
	IgM		5.7		2.2						2.6		2.9							*8.1*
4	IgG	41.3 +	22.5 +	+	55.8 +	13.7 +	+	−	10.7 +	114.9 +	−	−	−	−	−	+	−	+	−	3.2 +
	IgA	13.3	14.1	9.2	9.1	10.4				23.4		*66.0*	*15.7*							
	IgM	14.5							4.1											21.0
5	IgG	+	+	1.7 +	+	+	1.2 +	*1.4* −	1.4 +	+	−	−	−	+	*1.4* −	+	−	+	−	1.1 +
	IgA											*1.8*								1.3
	IgM				2.8				5.3						*5.2*					1.6
6	IgG	*1.5* −	1.3 +	+	+	1.5 +	1.6 +	+	1.2 +	+	1.2 +	+	+	−	1.2 +	*1.3* −	*1.2* −	−	+	*1.6* −
	IgA	*1.3*	1.3	1.3	1.3	1.7	1.4		1.7											
	IgM				1.7			5.9												
7	IgG	−	+	+	+	+	+	−	6.9 +	5.8 +	+	+	+	−	−	+	+	+	−	−
	IgA	*1.3*	1.6	1.3	1.6				7.5											
	IgM				1.6	1.4	2.4	*1.7*	10.0											
8	IgG	+	1.3 +	+	1.1 +	+	+	−	1.1 +	1.5 +	−	−	+	−	−	+	+	+	−	−
	IgA	1.8	1.7						2.8											
	IgM	23.9						*1.5*	1.4											
9	IgG	−	+	+	+	+	1.6 +	−	2.9 +	5.1 +	64.7 +	+	+	−	−	+	3.1 +	+	−	−
	IgA						1.9		1.9	5.8							8.2			
	IgM																			
10	IgG	+	+	2.2 +	+	+	−	−	1.7 +	4.7 +	−	3.5 +	2.5 +	−	−	−	−	−	−	*2.7* −
	IgA		1.5							2.3										
	IgM	1.2			4.6				3.6	3.4			9.8							
11	IgG	1.1 +	+	1.5 +	2.2 +	1.7 +	+	+	1.5 +	+	2.3 +	+	+	−	+	+	+	+	−	−
	IgA			1.2	1.8															*1.8*
	IgM																			
12	IgG	1.2 +	+	+	+	+	2.0 +	1.8 +	+	+	2.0 +	2.0 +	*2.3* −	−	−	−	−	−	−	−
	IgA			1.6		1.4	1.6		1.3											

	IgM																		
13	IgG	–	1.1 +	2.7 +	2.3 +	1.9 +	–	–	1.1 +	2.5 +	–	1.6 +	2.7 +	–	–	–	–	–	–
	IgA		2.0	1.2	1.3	3.9			1.9	2.0									
	IgM																		

Empty cells indicate that there is no increase. If there was an increase in antibody level, but the corresponding gene was not detected in the *S. aureus* isolate, the fold increase is noted in *italics*. Peak IgG level was not reached for FnbpA in patient 2, FnbpA and IsdA in patient 5, Efb in patient 11, and Efb, ClfB, SdrD and SdrE in patient 13. Here, the fold increase from initial to highest IgG level is noted

\+ The corresponding gene was detected in the *S. aureus* isolate

– The corresponding gene was not detected in the *S. aureus* isolate

previously [21–26]. Furthermore, *S. aureus* strains were genotyped by pulsed-field gel electrophoresis (PFGE) [27].

Measurement of antistaphylococcal antibodies

The levels of IgG, IgA, and IgM in the serum of the patients directed against the MSCRAMMs, SEs, and immunomodulatory proteins were quantified using a bead-based flow cytometry technique (xMAP, Luminex Corporation). This technique allows for the quantification of antibodies in small serum volumes to 19 antigens simultaneously. For IgG and IgA measurement, sera were diluted 1:100. For IgM measurement, sera were diluted 1:25. The methods were as described previously [16, 28, 29]. Tests were performed in independent duplicates and the median fluorescence intensity (MFI) values, reflecting antibody levels, were averaged. In each experiment, control beads (no protein coupled) were included to determine non-specific antibody binding. In case of non-specific binding, these non-specific MFI values were subtracted from the antigen-specific results. Human pooled serum (HPS) was used as a standard.

Statistical analysis

Statistical analyses were performed with SPSS version 15.0. To compare the initial antibody level with the peak antibody level, the Wilcoxon matched-pairs signed-ranks test was used. $p<0.05$ was considered as statistically significant.

Results

PFGE analysis

PFGE analysis was performed for all *S. aureus* strains isolated. A dendogram of the PFGE data (Fig. 1) shows the overall lack of relatedness among the strains from different patients, with the exception of patients 4 and 5. There was no epidemiological relationship between these two patients. All *S. aureus* strains isolated from an individual patient were genotypically indiscriminate (>95% relatedness) and clustered in the dendogram; only in the case of patient 9 were the PFGE patterns of the strains slightly different.

Detection of virulence genes

Based on PCR analyses, *clfA*, *clfB*, and *isdA* were ubiquitous in all 44 isolates obtained from the 15 bacteremic patients. *scn*, *efb*, *fnbA*, and *isdH* were detected in >90% of the isolates. *sdrD*, *sdrE*, *sasG*, *sei*, *seo*, *sem*, *chp*, and *fnbB* were detected in 35 (80%), 33 (75%), 30 (68%), 28 (64%), 28

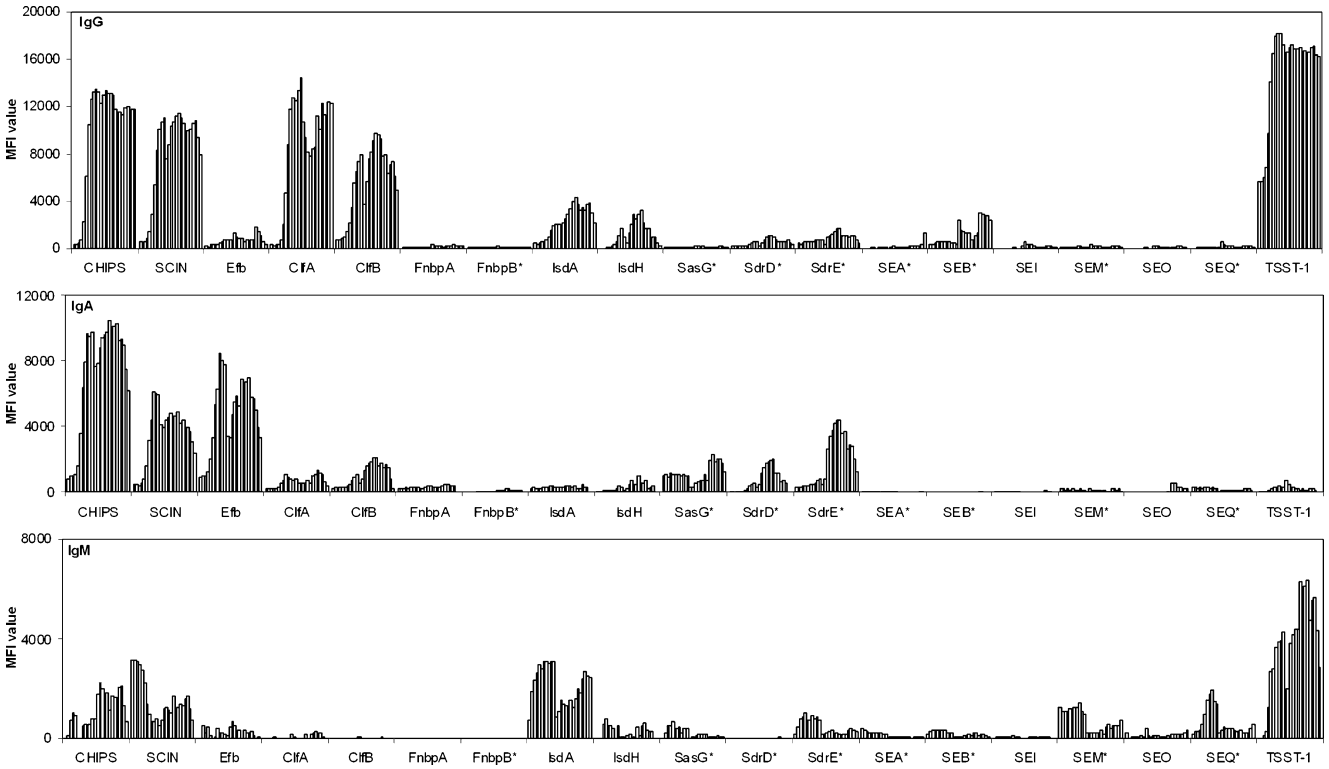

Fig. 2 Course of IgG, IgA, and IgM levels directed to 19 *S. aureus* proteins following bacteremia. The results are shown for patient 4. Each bar represents a serum sample on a separate day. The time period between the first and last serum sample is 58 days. *The corresponding gene was not detected in the *S. aureus* isolate

(64%), 23 (52%), 23 (52%), and 14 (32%) of the isolates, respectively. Less than 20% of the isolates harbored *sea*, *seb*, *seq*, and *tst-1*. Overall, all strains isolated from a single patient (PFGE-identical) harbored the same virulence genes, with two exceptions. In patient 6, *scn* was detected only in the strain isolated from foot tissue, but not in the strains isolated from blood and the superficially cultured wound of the foot. In patient 15, *sasG* was detected only in the strain isolated from catheter-drawn blood, but not in the strain isolated from peripheral blood or bone marrow (Fig. 1). An overview of the presence of virulence genes in *S. aureus* strains per patient is shown in Table 2.

Antistaphylococcal antibodies

The levels of IgG, IgA, and IgM directed to 19 *S. aureus* proteins were measured from diagnosis bacteremia onwards. In Fig. 2, the changes in antistaphylococcal IgG, IgA, and IgM levels after diagnosis bacteremia are shown for one patient (patient 4). For all patients, the course of the antibody response was determined. If there was an increase in antibody level in the consecutive serum samples, the fold increase from initial antibody level (as measured in the first obtained serum sample) to peak level was calculated. An overview for the 13 adult patients is shown in Table 2. In each patient, increases in antistaphylococcal antibody levels directed to different *S. aureus* proteins were observed. Antibody responses were directed mainly to the immunomodulatory proteins and MSCRAMMs. An increase in anti-IsdA IgG level was detectable in 12 out of 13 (92%) adult bacteremic patients (Fig. 3; with the exception of patient 12). Some patients showed a small decline in anti-IsdA IgG level before the increase. The median increase from initial to peak level was 1.71-fold (range 1.13–10.70-fold, $p<0.01$). The number of days to reach anti-IsdA IgG peak levels ranged between 7 and 37 days (median 21 days). It appeared that patients with a site of infection other than catheter-related (patients 6, 7, 8, 11, and 13) reached higher levels, but the anti-IsdA IgG peak level was high in patient 2 as well (Fig. 3). In Table 3, the dynamics of the IgG responses directed against all immunomodulatory proteins and MSCRAMMs are summarized per *S. aureus* protein.

An increase in antigen-specific IgA was detected less frequently than an increase in IgG. In 7 out of 13 patients with a gene-positive isolate, the anti-IsdA, anti-SCIN, and anti-Efb IgA level increased. Peak levels were reached between 6 and 34 days. In not a single patient was a rise in anti-FnbpB, SasG, SEA, SEB, SEO, or SEQ IgA level detected. Increases in IgM were detected the least often out of the three antibody isotypes

(Table 2). For patients 9 and 11, most antigen-specific IgM levels showed a decrease.

In the two children, the antibody responses were quite different from the antibody responses in adults. In the 5-day-old newborn (patient 14), a continuous decline in antigen-specific (maternal) IgG for 18 out of 19 proteins was seen from the moment of diagnosis. This could be due to the extensive consumption of maternal antibodies in the absence of de novo antibody synthesis (Fig. 4). The level of IgM increased for CHIPS (isolate *chp*-negative), SCIN, SasG, SdrD, SdrE, SEM, SEO, and SEQ (*seq*-negative). The level of IgA was around zero. In the 2-year-old child (patient 15), an increase was noted for IgG directed to IsdA and CHIPS only. IgM increased for SCIN, ClfA, SdrD, SdrE (isolate *sdrD*- and *sdrE*-negative), SEI, SEM, SEO, and TSST-1 (*tst*-negative).

In the four control patients who did not suffer from an *S. aureus* bacteremia, the CNS-infected patient showed a 1.2-fold increase in anti-CHIPS IgG level (from MFI 13333 to MFI 16002), and the *K. pneumoniae*-infected patient showed a 1.4-fold increase in anti-ClfB IgG level (from MFI 5647 to MFI 8058). Furthermore, no increases in antistaphylococcal antibody levels were noted. This shows that there is little inter-species cross-reactivity. However, in the patients suffering from an *S. aureus* bacteremia, a rise in antibody level while the corresponding gene was not present in the *S. aureus* isolate was observed in 23 of 179 increases (12.8%; Table 2).

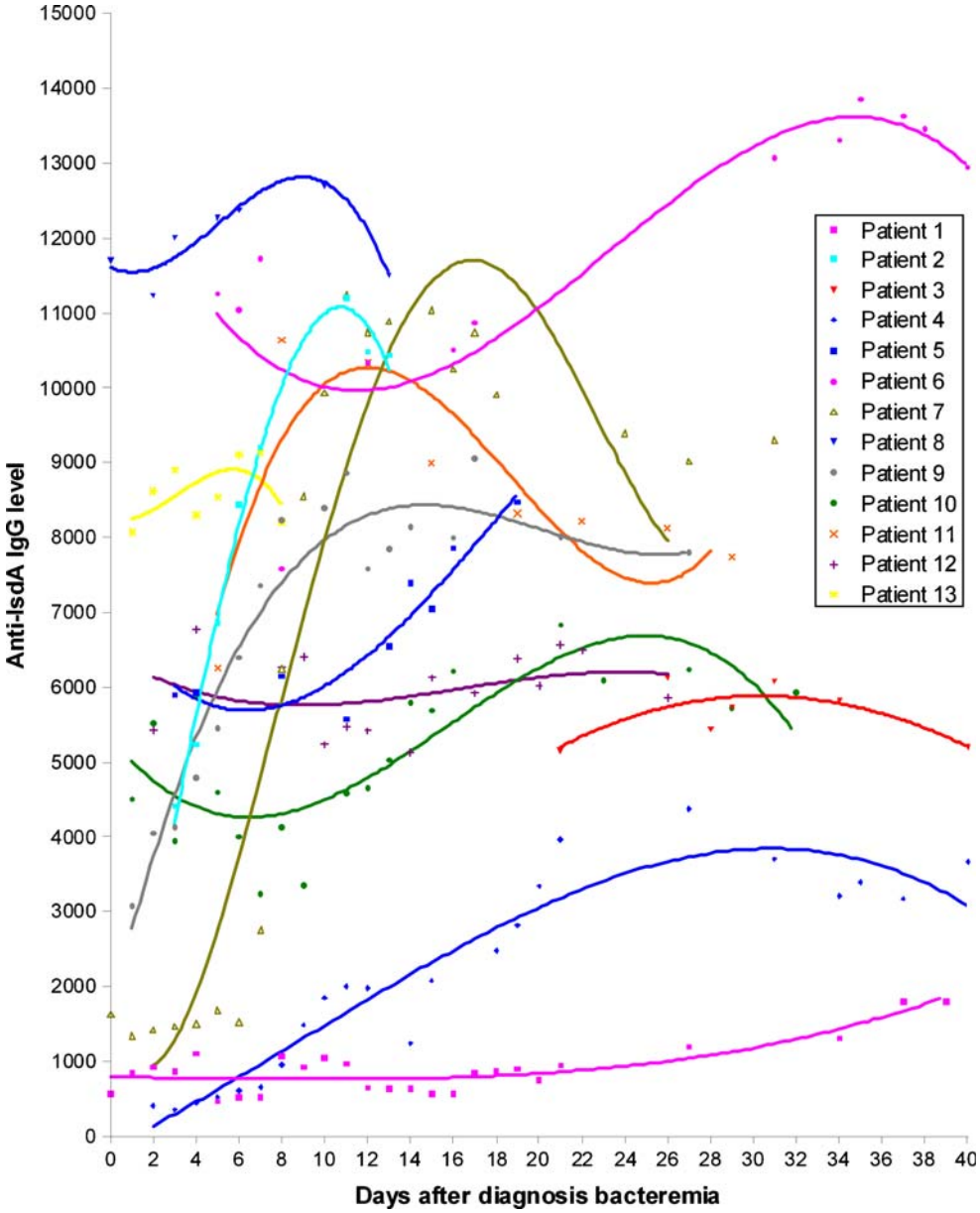

Fig. 3 Course of anti-IsdA IgG levels following *S. aureus* bacteremia in different adult patients

Table 3 Dynamics of the antistaphylococcal IgG response during bacteremia

Protein	No. of adult patients with gene-positive isolates (%)	No. of adult patients with increase in IgG level (%)	Median fold increase from initial to peak level (range)[a]	Median no. of days to reach peak level (range)[a]	*p*-value[d]
CHIPS	9/13 (69%)	4/9 (44%)	2.9 (1.1–41.3)	25 (11–27)	–
SCIN	13/13 (100%)	5/13 (38%)	1.3 (1.1–22.5)	21 (5–42)	<0.05
Efb	12/13 (92%)	6/12 (50%)	1.7 (1.5–2.1)	24 (14–44)	<0.05
ClfA	13/13 (100%)	6/13 (46%)	1.9 (1.1–55.8)	19 (10–40)	<0.05
ClfB	13/13 (100%)	7/13 (54%)	1.5 (1.2–13.7)	28 (11–50)	<0.05
FnbpA	11/13 (85%)	7/11 (64%)	1.6 (1.4–2.0)	27 (11–42)	<0.05
FnbpB	4/13 (31%)	1/4 (25%)	1.8[b]	23[c]	–
IsdA	13/13 (100%)	12/13 (92%)	1.7 (1.1–10.7)	21 (7–35)	<0.01
IsdH	12/13 (92%)	7/12 (58%)	4.7 (1.5–114.9)	15 (7–34)	<0.05
SasG	6/13 (46%)	4/6 (67%)	2.1 (1.2–64.7)	23 (11–42)	–
SdrD	10/13 (77%)	5/10 (50%)	4.7 (2.0–19.4)	22 (11–27)	<0.05
SdrE	10/13 (77%)	3/10 (30%)	2.0, 2.5[b]	11, 21[c]	–

[a] Peak IgG level was not reached for FnbpA in patient 2, FnbpA and IsdA in patient 5, Efb in patient 11, and Efb, ClfB, SdrD, and SdrE in patient 13; therefore, these data were excluded

[b] Absolute fold increase in IgG level

[c] Absolute number of days until peak level

[d] *p*-values<0.05 (by the Wilcoxon matched-pairs signed-rank test) were considered to be statistically significant

Discussion

By using multiple, longitudinally collected serum samples, we were able to show that each bacteremic patient develops a unique *S. aureus*-specific immune response after infection. In each bacteremic patient, the *S. aureus* proteins to which the antibody response was directed differed and the height and number of days to reach peak antibody level differed. Therefore, it seems unlikely that a vaccine based on a single staphylococcal component will be effective. There are several possible explanations for the variability of the antistaphylococcal immune response in bacteremic patients. First, it may be the result of the genetic diversity of the *S. aureus* strains which caused the bacteremia [30]. Secondly, the variability might be due to different staphylococcal protein expression and/or selective protein recognition by the immune system in different patients. By way of illustration, although patients 4 and 5 were infected with PFGE-identical *S. aureus* strains with similar virulence genes, the antibody responses were very different (Fig. 1 and Table 2). The observed variability in antibody response might also be caused by the difference in the time of onset of the bacteremia, which is difficult to determine accurately for patients with an origin of bacteremia other than catheter-related. Finally, colonization, the number and severity of preceding infections, the level of immunity, and the ability to mount an immune response probably also contribute to the development of the antistaphylococcal humoral immune response after bacteremia [31].

Although the antistaphylococcal immune response after infection is a unique personal characteristic, IsdA is immunogenic and, therefore, produced by *S. aureus* in nearly all bacteremic patients. IsdA interacts with and binds to fibrinogen and fibronectin of human cells [32], and is involved in the adherence of *S. aureus* to human desquamated nasal epithelial cells [33]. Furthermore, IsdA is required for nasal colonization in the cotton rat model [33]. Antibodies reactive to IsdA enhanced the killing of *S.*

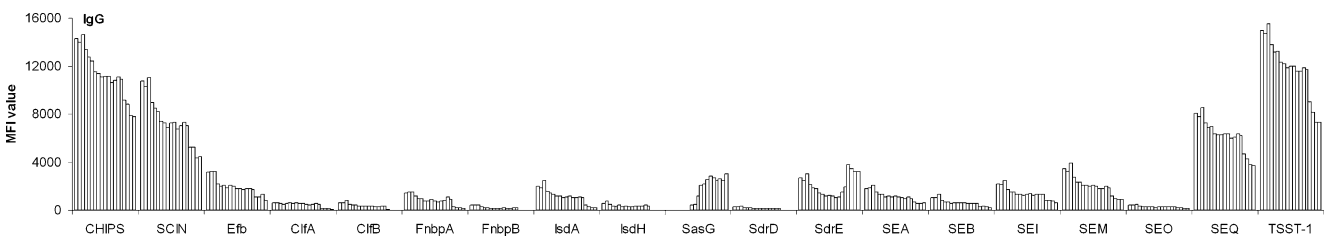

Fig. 4 Course of IgG levels directed to 19 *S. aureus* proteins in a 5-day-old bacteremic newborn (patient 14). Each bar represents a serum sample on a separate day. The time period between the first and last serum sample is 32 days

aureus by the antimicrobial peptide apolactoferrin in vitro [34]. Since vaccines should contain multiple components that are expressed in vivo in many, if not all, patients, it seems that IsdA is an interesting target for inclusion in a multicomponent vaccine [35].

In 23 of 179 (12.8%) observed increases, a rise in the antistaphylococcal antibody level was found while the corresponding gene was not detected in the *S. aureus* isolate. Increasing anti-TSST-1, anti-SEB, and anti-FnbpB antibody levels in the absence of *tst*, *seb*, and *fnbB* genes were documented most frequently (Table 2). This might be the result of immunological cross-reactivity. In earlier studies, it was shown that anti-SEB cross-reacts with anti-SEC1 [36, 37]. Furthermore, it was shown that anti-TSST-1 also has cross-inhibitory activity against SEA and SEB [38, 39]. However, the relative binding affinity was 1,000-fold lower for SEA than for TSST-1 and, therefore, this does not seem to be clinically relevant. Another explanation might be that the antibodies are secreted by plasma cells that are mobilized from their survival niche in the bone marrow by competition with newly generated plasma blasts [40]. Knowledge on non-specific antibody binding should be increased, however.

In summary, bacteremic patients usually carry a PFGE-identical *S. aureus* strain at multiple sites. Each bacteremic patient develops a unique immune response directed against different *S. aureus* proteins. Therefore, a vaccine based on a single antigenic component is not likely to be effective. The surface protein IsdA is immunogenic and, therefore, produced by *S. aureus* in nearly all bacteremic patients. This suggests that IsdA might be a useful component of a multivalent staphylococcal vaccine.

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