Staphylococcus aureus Clonal Dynamics and Virulence Factors in Children with Atopic Dermatitis

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A prospective cohort study was undertaken to determine the clonal dynamics of *Staphylococcus aureus* colonization and infection during 1 y in children with atopic dermatitis, and to correlate specific clones, *accessory gene regulator* (*agr*) groups, and production of virulence factors with eczema activity. Eleven children were examined every 6 wk with swaps taken from active eczema, anterior nose, axillae and perineum, and scoring of eczema activity by severity scoring of atopic dermatitis (SCORAD). Individual *S. aureus* clonal types were identified and examined for production of superantigens, toxins, and were assigned to *agr* groups. *S. aureus* colonization patterns ranged from rare colonization over transient colonization to persistent colonization by a single clone or a dynamic exchange of up to five clones. Production of no single virulence factor including superantigens and toxins was significantly associated with exacerbation of eczema. In four children there was a shift between visits in *agr* group of colonizing clones. These shifts were associated with an increased SCORAD value of 19 (SE = 7, p = 0.009). Change of clones belonging to the same *agr* group was not associated with a higher SCORAD value. In 11 of 12 cases with two different clones co-colonizing a child the clones belonged to the same *agr* group. In conclusion, this limited group of children with atopic dermatitis showed highly variable colonization patterns of *S. aureus*, and communication between strains by use of *agr* encoded octa peptides appeared to be active *in vivo*. Increased severity of eczema was related to a change in *agr* group and may have been because of inflammation triggered by the takeover of an antigenically different clone, as *agr* groups represent ancient phylogenetic lineages.

Key words: *agr*/atopic dermatitis/*Staphylococcus aureus*/superantigen/toxin
J Invest Dermatol 125:977–982, 2005

Children with atopic dermatitis are more frequently colonized by *Staphylococcus aureus* on both lesional and nonlesional skin than healthy children. A higher density of *S. aureus* in lesional skin is associated with more severe eczema, but to what extent and how the staphylococci aggravate the eczema is not clear. *S. aureus* produce a multitude of factors with potential skin inflammatory properties including adhesins, cytotoxins, superantigens, capsule, epidermolytic toxins, and a number of extracellular enzymes. In general, it is assumed that staphylococcal infection leads to a worsening of atopic eczema and that antibacterial treatment is beneficial when the children are clinically impetiginized. The role of antibiotics is more controversial when the skin is only colonized and not clinically infected (Williams, 2000; Lübbe, 2003).

The population of *S. aureus* is comprised of many clonal lineages that produce different combinations of virulence factors (Peacock *et al*, 2002). Recent findings indicate that clones of *S. aureus* are able to communicate during co-colonization by means of small octa peptides (pheromones) encoded by the *accessory gene regulator* (*agr*) gene and interfere with the colonization of competing clones by bacterial interference (Yarwood and Schlievert, 2003). When the concentration of the *agr*-encoded octa peptide is high, exotoxin production is upregulated whereas surface molecules including adhesins are downregulated. Four *agr* groups have been recognized based on sequence variation in the *agr* gene (Ji *et al*, 1997) and these groups appear to reflect separate phylogenetic lineages (Jarraud *et al*, 2002). Members of the same group stimulate each other, whereas members of different groups are mutually inhibitory with the exception of groups IV and I, where the interaction is not entirely clear. Only few data are available on the significance of this system *in vivo* and it is not known whether it plays a role in the colonization of atopic dermatitis.

For a better understanding of the interplay between staphylococci and the atopic host, it is important to examine in detail the clonal colonization dynamics over time. Therefore, the present longitudinal study was undertaken in children with atopic dermatitis followed for approximately 1 y. Clones were discerned using pulsed-field gel electrophoresis (PFGE), screened for a number of putative virulence factors, and assigned to one of the four *agr* groups. Clonal characteristics were compared with eczema activity at the time of colonization. Although the number of children was limited, the results suggest that the *agr*-related diffusion sensing system in an atopic dermatitis patient plays an

Abbreviations: *agr*, *accessory gene regulator*; PFGE, pulsed-field gel electrophoresis; SCORAD, severity scoring of atopic dermatitis; SEA, SEB, etc., staphylococcal enterotoxin A, B, etc.

The work was performed in Odense and Aarhus, Denmark.

important role in determining the colonization and hence eczema activity over time.

Results

***S. aureus* colonization patterns and eczema** In total the 11 children were seen on 95 visits and 378 swaps were obtained, 162 of which yielded growth of *S. aureus*. From many swaps *S. aureus* colonies with more than one colony morphology could be distinguished and a total of 240 different colonies were isolated. *S. aureus* were recovered from the perineum on 26 visits, eczema on 58 visits, nose on 55 visits, axillae on 23 visits, and no *S. aureus* were recovered on 25 visits.

The 240 *S. aureus* isolates were assigned to different clones based on the DNA fingerprint pattern revealed by PFGE. Isolates exhibiting identical band patterns were considered belonging to the same clone as suggested (Tenover *et al*, 1995). Successive clones from each child were aligned on gels and eventually a total of 28 representative clones were distinguished considering clones from different children as separate clones (Fig 1). By grouping together possibly related clones differing in less than six bands (as defined by Tenover *et al*, 1995) eight groups of unrelated clones could be defined (not shown). Two pairs of siblings shared identical clones (Table I, child 2 clone A and child 3 clone A, and child 10 clone B and child 11 clone A).

As given in Table I the 11 children showed variable patterns of *S. aureus* colonization during the study period. On 16 occasions two clones colonized the same child simultaneously and on two occasions three clones co-existed. In most cases the co-colonizing clones were isolated from different locations, but two simultaneous clones were found three times in the nose, two times in eczema, and once in the perineum.

Twenty courses of systemic antibiotics were administered during the study period (Table I) often in combination with local fucidin, chinoform containing topical steroid, or potassium permanganate soak, and once with mupirocin nasally. All clones were susceptible to methicillin and erythromycin. Thirteen clones were relatively resistant to fucidic acid, but the concentration following topical use on skin *in vivo* is thought to be far above the minimal inhibitory concentration. As shown in Table I *S. aureus* clones generally persisted or returned in spite of antibiotic and antiseptic treatment, and neither antibiotic treatment nor the use of local steroids seemed to influence clonal shifts.

When data were analyzed using a longitudinal autoregressive model, simultaneous *S. aureus* colonization at all four sites was significantly associated with an increased severity scoring of atopic dermatitis (SCORAD) value of 17 ($SE = 6$, $p = 0.006$) (Table II). In this respect, four children showed simultaneous colonization at all four sites on two, five, three, and four occasions, respectively. Moreover, there was a clear positive trend towards a higher SCORAD value the more of the sites that were colonized, indicating that increased *S. aureus* colonization was associated with more severe eczema. Colonization at no single site was correlated with significantly more eczema except for the perineum. *S. aureus* at this location, however, was highly correlated to the simultaneous appearance of the organism at the other sites, so it probably just reflected widespread colonization.

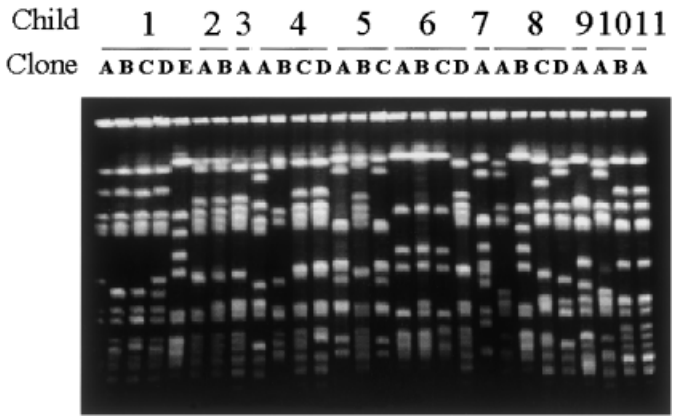

Figure 1
Pulsed-field gel electrophoresis patterns of 28 clones identified from 11 children with atopic dermatitis. From each child successive clones are numbered alphabetically. Clones from different children with the same designation are not identical.

***Agr* groups and eczema** The 28 clones detected were assigned to the four known *agr* groups with a distribution among groups as given in Table III. In 17 of 18 cases clones co-colonizing the same patient shared *agr* group. The exception was in patient 8, where clone D assigned to *agr* group II and clone C assigned to *agr* group I simultaneously colonized the anterior nose of the patient at one occasion. If a particular combination of two clones was counted only once for each patient, the corresponding numbers were 11 of 12 cases. If the frequency of each *agr* group in Table III is used as the probability of being colonized with strains from that particular group, then the probability of being colonized with strains belonging to the same *agr* group can be estimated as $(0.46^2 + 0.18^2 + 0.32^2 + 0.04^2) = 0.3480$. Then the distribution of co-colonizing strains belonging to the same *agr* group in 11 of 12 cases does not seem to be a random coincidence (probability = 0.000074, binomial distribution).

Table I shows a shift in overall *agr* group in four patients associated with a significant increase in SCORAD value of 19 ($SE = 7$, $p = 0.009$) (Table II). In contrast, a shift in clones within the same *agr* group was significantly associated with a small decrease in SCORAD 11 ($SE = 5$, $p = 0.03$). A shift in clones belonging to different clone groups, but of the same *agr* group, was not significantly associated with changes in SCORAD.

Adhesins, cytotoxins, superantigens, and eczema The 28 representative *S. aureus* clones were tested for a number of putative virulence factors including binding proteins, cytotoxin, epidermolytic toxins, and superantigens. The number of clones positive for each of these factors is given in Table III. No clone was found to produce more than one of the superantigens. The most prevalent superantigen and toxin were staphylococcal enterotoxin A (SEA) and β toxin, and the combined production of these was found in seven clones. For each child the production of superantigens and toxins at the visits is given in Table I in relation to clonal type and eczema activity. When these data were

Table I. Patterns of *Staphylococcus aureus* clonal colonization, virulence factors, eczema activity, and antibiotic treatment in 11 children with atopic dermatitis examined at six to 11 occasions during approximately 1 y

Child no.	Pattern[a]	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10	Visit 11
1	Clone	AB	AB	BCD	BE	BD	D	B	BD	BDE		
	SCORAD	38	47	30	32	48	41	50	44	41		
	Antibiotic					D						
	Factors				SEC					SEC		
2	Clone	AB	A	A	A	A	A	A	A	A		
	SCORAD	83	49	72	49	73	63	41	75	72		
	Antibiotic	D				Z	Z					
	Factors	SEA, β	SEA, β	SEA, β	SEA, β	SEA, β	SEA, β	SEA, β	SEA, β	SEA, β		
3	Clone	A	—	A	A	A	—	—	—	—		
	SCORAD	35	12	16	45	40	19	49	49	24		
	Antibiotic	D		D								
	Factors	SEA, β		SEA, β	SEA, β	SEA, β						
4	Clone	AB	A	AB	AB	A	**C**[b]	**C**	**C**	**CD**		
	SCORAD	71	58	51	78	78	92	55	55	56		
	Antibiotic				D		Z	Z D				
	Factors	SEB		SEB	SEB		SEA	SEA	SEA	SEA		
5	Clone	A	AB	AB	C	B	A	A	AB			
	SCORAD	55	44	28	23	40	30	58	41			
	Antibiotic											
	Factors		SEA, β	SEA, β		SEA, β			SEA, β			
6	Clone	A	A	A	AB	A	AC	C	**D**	**D**		
	SCORAD	32	53	31	40	47	36	16	42	50		
	Antibiotic					Z				D		
	Factors											
7	Clone	A	A	A	—	A	—	A	A	A		
	SCORAD	23	25	49	13	47	20	35	10	12		
	Antibiotic											
	Factor	ETA + B	ETA + B	ETA + B		ETA + B		ETA + B	ETA + B	ETA + B		
8	Clone	—	A	A	**B**	**B**	**B**	**CD**	—	—	—	**C**
	SCORAD	Nd	19	11	54	64	70	31	42	65	37	18
	Antibiotic				D	D	E	E D	D	D		
	Factors											
9	Clone	—	—	A	—	—	A					
	SCORAD	56	55	46	54	35	27					
	Antibiotic	E										
	Factors			SEA, β			SEA, β					
10	Clone	—	A	**B**	**B**	—	**B**	—	**B**			
	SCORAD	21	36	61	53	29	37	34	24			
	Antibiotic											
	Factors			SEA, β	SEA, β		SEA, β		SEA, β			
11	Clone	—	—	—	—	—	—	—	A			
	SCORAD	24	21	53	16	22	Nd	8	24			
	Antibiotic			D								
	Factors								SEA, β			

[a]Clone: the first clone identified in each child is arbitrarily designated A, the second B, the third C, etc. Clones from different children with the same designation are not identical. Antibiotic: courses of systemic antibiotics are indicated when administered, D represents dicloxacillin, Z azithromycin, and E erythromycin. Factors: production of the superantigens staphylococcal enterotoxin A, B, and C (SEA, SEB, and SEC), epidermolytic toxins A and B (ETA + B), and β toxin (β).

[b]Bold figures and gray shades indicate clones of an *agr* group different from the former clones colonizing the child.

SCORAD, severity scoring of atopic dermatitis; *agr*, *accessory gene regulator*.

Table II. Effect on the eczema activity by *Staphylococcus aureus* colonization, *agr* group shift, and change in clones as calculated by means of a longitudinal autoregressive model

Variable	Effect on SCORAD	Standard error	p-value
Agr group shift	19	7	0.009
Clone shift within			
Agr groups	−11	5	0.03
S. aureus colonization at			
No site	0	—	—
One site	−3	4	0.51
Two sites	7	5	0.12
Three sites	9	6	0.13
Four sites	17	6	0.006

SCORAD, severity scoring of atopic dermatitis; *agr*, *accessory gene regulator*.

applied to the statistic model none of these factors were associated with a significant increase in SCORAD value, neither when calculated separately for each of the four sampling sites nor when all sites were combined.

Discussion

This study confirmed the abundant colonization of atopic eczema patients with *S. aureus*. Staphylococcal colonization at all four sites was associated with a significantly higher value of SCORAD 17 (SE = 6, p = 0.006) and a solid trend was found towards more active eczema the more of the sites that were colonized. Previous studies have shown that the number of *S. aureus* on the skin is positively correlated to the activity of atopic dermatitis (Williams *et al*, 1990). But the cause relationship in these findings is not known.

To study the interplay between *S. aureus* and eczema over time the clonal dynamics during colonization was examined in detail by using chromosomal DNA fingerprinting. Several different patterns of colonization were revealed with some children continuously harboring a single persistent clone, whereas others experienced a dynamic exchange of up to five clones colonizing different or the same location during the observation period. An intermittent pattern of colonization was observed in several children and one child was colonized on only a single occasion. Although the number of children is limited, these findings agree with the three carriage patterns reported in healthy carriers: noncarriers, intermittent carriers, and persistent carriers, with persistent carriage being more frequent among children (Kluytmans *et al*, 1997; Vandenbergh and Verbrugh, 1999).

The finding that two pairs of siblings shared clones indicates that spread within families takes place as previously reported (Breuer *et al*, 2002). Confounding because of siblings cannot be entirely ruled out. But this is probably a minor problem because exclusion of the four siblings in the analysis revealed a similar increase in SCORAD value 17 (SE = 8, p = 0.05) associated with *agr* group shift. Within sibling pairs the shared clones were carried in different patterns over time so that the effect of virulence factors on eczema activity might be expected to occur independently in each child and from common factors in their environment.

Table III. Virulence factors detected among 28 *Staphylococcus aureus* clones

Virulence factor	Number of positive strains	Percentage of positive strains
Superantigens		
TSST-1	0	0
SEA	8	29
SEB	1	4
SEC	1	4
SED	0	0
Cytolytic toxins		
β toxin	7	25
Epidermolytic toxins		
ETA	1	4
ETB	1	4
Adhesins		
fnbA	28	100
cna	15	54
Agr groups		
Agr I	13	46
Agr II	5	18
Agr III	9	32
Agr IV	1	4
Haemolytic activity		
+	10	36
++	4	14
+++	14	50
Proteolytic activity		
None	4	14
+	2	7
++	8	29
+++	14	50

TSST-1, toxic shock syndrome toxin 1; SEA, SEB, and SEC, staphylococcal enterotoxin A, B, and C; *agr*, *accessory gene regulator*.

We initially hypothesized that the appearance of a new clone with a new combination or antigenic makeup of virulence factors leads to a flare of eczema. But no exacerbation of dermatitis was associated with clonal exchanges as long as the clones belonged to the same *agr* group. In fact, statistical analysis showed a decrease in SCORAD 11 (SE = 5, p = 0.03). When calculated as a change to another of the eight clone groups defined by PFGE analysis, possibly reflecting a change to a genetically more distant strain than mere clone shifts, there was no significant change in SCORAD.

To investigate whether bacterial interference among colonizing strains of *S. aureus* plays a role *in vivo* in atopic dermatitis all isolates were assigned to one of the four *agr* groups. The overall distribution of *agr* groups was similar to findings reported for isolates from healthy nasal carriers and patients with cystic fibrosis (Goerke *et al*, 2003; Kahl *et al*, 2003). The finding that in 11 of 12 instances, where more than one clone colonized a child, the co-colonizing clones belonged to the same *agr* group (probability of mere coincidence = 0.000074) suggests that bacterial interference is active *in vivo* during *S. aureus* colonization of atopic eczema.

Interestingly, among four of the children followed in this study a shift from one *agr* group to another was observed between two visits. These *agr* group shifts were associated with a significant flare of eczema 19 (SE = 7, p = 0.009). Of note, three of the four *agr* group shifts were preceded by improvement in eczema, but followed by a subsequent flare. One might speculate that a low number of colonizing *S. aureus* lead to downregulation of virulence factors by negative quorum sensing (Novick, 2003) in the existing strain with ensuing facilitated settlement and spread of the newcomer. As the *agr* groups appear to reflect ancient phylogenic lineages, the newcomer conceivably produces a new set of antigenically significantly different virulence determinants. Because of a lack of neutralizing antibodies, increased inflammation may result. The subsequent decrease in eczema to "base-line" activity may be because of the eventual induction of such antibodies.

All isolates were examined for a number of adhesins, toxins, and superantigens to study the possible effect of single virulence factors in aggravating dermatitis. No clear association was found between production of superantigens and/or β toxin and increased eczema. Two cross-sectional studies have shown more severe eczema in adults and children colonized with superantigen-producing *S. aureus* as compared with superantigen negative strains (Bunikowski *et al*, 2000; Zollner *et al*, 2000). Two other studies, however, did not confirm this association (Arkwright *et al*, 2001; Mempel *et al*, 2003). The interplay between *S. aureus* virulence factors and eczema is highly complex and although the present study did not support a role for superantigens and/or β toxin, it does not rule out an effect of these in exacerbating atopic eczema under certain circumstances. It is conceivable that the number of bacteria colonizing a surface is crucial. Also, recent findings indicate that the total diversity of *S. aureus* superantigens may be extreme (Kuroda *et al*, 2001) with many more different superantigens than the classical ones examined so far.

In conclusion, the children with atopic dermatitis showed variable patterns of colonization with *S. aureus*: a few are rarely, some intermittently, and some persistently colonized. Persistent colonization may be with a single clone or a dynamic exchange of several clones. Colonization with *S. aureus* was associated with more severe eczema, whereas no single virulence factor including the classical superantigens was associated with exacerbation of eczema. Bacterial interference via the *agr* diffusion sensor appears to play a role determining clonal dynamics *in vivo* with a change in *agr* group among colonizing clones significantly associated with a flare in eczema. The sample size of the present study is limited and future studies should be directed at detailed examinations of the interplay between the host immune and inflammatory systems and bacterial virulence factors at the time of shifts in *agr* groups to confirm and expand the present findings.

Materials and Methods

Eleven children aged 2–11 y, eight boys and three girls, with mild-to-severe atopic dermatitis diagnosed according to the criteria of Hanifin and Rajka (1980) were each followed for a mean of 12 months (range 11–14) at the Department of Dermatology, Odense University Hospital, Denmark, in the period 2001–2002. Two pairs of siblings were included. Consecutive children with atopic dermatitis were included after the parents gave written informed consent. The children were seen approximately every 6th wk and whenever acute exacerbations of the eczema occurred. Treatments were administered according to usual procedures at the department with local corticosteroids, local antiseptics, or antibiotics, and courses of systemic antibiotics when needed.

The study protocol was approved by the Ethics Committee of the Funen County, and the study was conducted according to the Declaration of Helsinki Principles.

Bacterial samples and clinical evaluation At every visit four swaps were taken with a coal impregnated cotton swap from the anterior nose, axillae, a representative area of active eczema, and the perineum. Samples were transported in Stuart's transport medium (Statens Serum Institut, Copenhagen, Denmark) and were received in the microbiological laboratory within 24 h for incubation on blood agar overnight at 37°C. Representatives of each colonial morphology of putative *S. aureus* were isolated and subcultured to purity for further analysis. The identity was confirmed by demonstrating coagulase activity.

The activity of atopic eczema was determined by using SCORAD (values 0–103) (European Task Force on Atopic Dermatitis, 1993).

Clonal analysis by pulsed-field gel electrophoresis Genomic DNA fingerprinting was done by PFGE using standard technology. Each *S. aureus* isolate was harvested from Todd-Hewitt broth (CM189, Oxoid, Basingstoke, Hampshire, UK) and lysed within the agarose (Bio-Rad Laboratories, Hercules, California) by incubation for 2 h at 37°C in a lysis buffer (TE10:100 buffer; 1% sakrosyl; 1.3×10^4 U per mL lysozyme, Roche, Basel, Switzerland; 100 U per mL mutanolysin, M9901, Sigma-Aldrich, Brønoby, Denmark) and subsequently treated in a solution of proteinase K (1 mg per mL, 705723, Roche; 0.5 M EDTA; 1% sarkosyl; pH 8.0) at 50°C for 14–20 h. After washing in Milli Q water, the DNA-containing agar plugs were incubated in 100 μL 1 × SurE/Cut buffer A (1417959, Roche) containing 10 U of *Sma*I restriction enzyme (656348, Roche) for 16 h at room temperature. Separation of DNA fragments was performed on a GenePath Strain Typing System using a CHEF-DRIII power module (Bio-Rad Laboratories) according to the manufacturer's recommendations and using the pre-programmed run condition "PSU" (initial ramp time, 5.3 s; final ramp time, 34.9 s; linear progression in ramp time; angle, 120° angel switching; voltage, 6 V per cm; running time 19.5 h). Strains that were compared were tested in the same gel. Banding patterns developed with ethidium bromide were recorded by photography.

Antibiotic susceptibility testing To examine if failure to eradicate clones of *S. aureus* from the skin of patients by antibiotic therapy was because of antibiotic resistance, all isolates were examined for susceptibility to methicillin, erythromycin, and fucidic acid using ROSCO susceptibility disks on blood agar (Rosco, Taastrup, Denmark).

Identification of *agr* alleles Allocation of all *S. aureus* isolates to *agr* groups I, II, III, and IV was done by detecting PCR products using the four primer pairs and PCR conditions described by Peacock *et al* (2002).

Detection of genes encoding toxin and adhesion proteins Gene sequences specific for exfoliative toxins A (*eta*) and B (*etb*) and the adhesins fibronection-binding protein A (*fnbA*) and collagen adhesin (*cna*) were detected by PCR using primer pairs and condition described by Peacock *et al* (2002).

Detection of toxin, hemolysin, and protease activity All isolates identified as *S. aureus* were examined for production of SEA, SEB, SEC, and SED and staphylococcal toxic shock syndrome toxin using the latex agglutination kits SET-RPLA and TST-RPLA according to manufacturer's instructions (Oxoid).

β-toxin activity was demonstrated by the ability of individual isolates to allow detection of the CAMP reaction (Christie *et al*, 1944*)* in an isolate of *Streptococcus agalactiae* on sheep blood agar purchased from Statens Serum Institute (Copenhagen, Denmark).

Proteolytic activity was demonstrated on skim milk agar inoculated with each strain in a line. The width of the clear zones developing in the medium around the bacterial growth after overnight incubation was rated (scores 0, 1, 2, and 3) to obtain a semiquantitative estimate of the proteolytic activity.

Statistical analysis In order to describe the association between clinical and microbiological parameters on the same child, the data were analyzed by means of a longitudinal model. The standard model to describe such a correlation is the compound symmetry model in which the correlation is independent of the spacing in time between measurements performed on the same child. In order to allow the correlation to depend on the spacing in time, an autoregressive model was preferred, where the correlation between two measurements is given by ρ^d, where d is the spacing in time (Diggle *et al*, 2002). Accordingly, because $-1<\rho<1$, the longer the time between two measurements, the weaker the correlation. The possible explanatory variables (*agr* group shift, clone shift, superantigen production, etc.) were added to the model in a stepwise manner in order to give a final "best" model. For the variables included in the final model, their presence was associated with either a positive or a negative change in the SCORAD value.

The authors thank Biostatistician Mogens Erlandsen, Department of Biostatistics Aarhus University, Denmark, for statistical analysis, and technician Tove Findahl for performing the laboratory work. The study was supported by the Danish Medical Research Council. The researchers are all independent from funders. This study was approved by the Ethics Committee of the Funen County.

DOI: 10.1111/j.0022-202X.2005.23916.x

Manuscript received February 27, 2005; revised July 5, 2005; accepted for publication July 7, 2005

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