Staphylococcus aureus Isolates from Patients with Kawasaki Disease Express High Levels of Protein A

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Kawasaki disease (KD) is an acute vasculitis of young children that can be complicated by coronary artery abnormalities. Recent findings suggest that a superantigen(s) may play an important role in stimulating the immune activation associated with the disease, although the origin of this superantigen(s) is unclear. Staphylococcus aureus, isolated from the rectum or pharynx of patients with KD, secretes toxic shock syndrome toxin 1 (TSST-1). The KD isolates express low levels of other exoproteins compared to isolates from patients with toxic shock syndrome (TSS). Thus, it was previously suggested that the KD isolates may be defective in the global regulatory locus agr (for accessory gene regulator), which positively regulates these factors (D. Y. M. Leung et al., Lancet 342:1385–1388, 1993). Here we describe another characteristic of KD isolates. When considered collectively, the KD isolates were found to express higher levels of staphylococcal protein A than the TSS isolates, another characteristic of an agr-defective phenotype. This correlated with a higher level of spa mRNA in these isolates. In contrast, the KD and TSS isolates expressed comparable levels of TSST-1, consistent with previous findings (D. Y. M. Leung et al., Lancet 342:1385–1388, 1993). Analysis of RNAIII transcript levels and nucleotide sequence analysis of the RNAIII-coding region suggested that the KD isolates are not defective in RNAIII, the effector molecule of the agr regulatory system. However, induction of RNAIII transcription in the KD isolates did not result in a dramatic decrease in the amount of spa mRNA, as has been reported for other strains (F. Vandenesch, J. Kornblum, and R. P. Novick, J. Bacteriol. 173:6313–6320, 1991).

toxin C-producing (n = 2) bacteria, compared with only 1 of 15 febrile controls. We proposed that TSST-1-producing Staphylococcus aureus and streptococcal pyrogenic exotoxin C-producing streptococci could account for at least some of the immune activation of T cells in the blood and tissues of patients with acute KD, which has now been reported by three independent groups (1, 2, 11, 33, 50). In a follow-up multicenter study, it was found that TSST-1-producing S. aureus could be isolated from 31 of 32 patients with acute KD (36). Staphylococcal protein A (SpA) is produced by over 95% of S. aureus strains and has the ability to bind to the Fc region of immunoglobulin G (IgG) in most mammalian species. This protein has been shown to inhibit opsonization and phagocytosis of staphylococci in vitro, a factor that may contribute to the virulence of this organism in vivo (15, 42, 43). SpA also exhibits diverse immunobiological properties, including an ability to activate B cells (B-cell superantigen) and complement (17, 26–28, 48). Interestingly, these laboratory parameters of immune activation have been reported during acute KD (24, 29). Thus, together with T-cell superantigens, such as TSST-1, SpA could account for at least some of the immune activation associated with this disease.

It was recently observed that S. aureus isolated from KD patients produced less lipase, hemolysin, and protease than isolates from TSS and other skin diseases. However, the KD isolates did not produce particularly low levels of TSST-1 (34). The expression of these exoproteins is controlled by global regulatory loci, including agr (for accessory gene regulator) (38, 44). This locus consists of two divergent transcripts, RNAII and RNAIII, driven by two distinct promoters, P2 and P3, respectively (40). RNAIII is the effector molecule of this
regulatory system. During the postexponential and stationary phases of growth, this molecule upregulates the expression of various exoproteins and downregulates the expression of factors such as Spa, the fibronectin-binding proteins, and coagulase (20, 41). In bona fide agr-defective mutants, there is a lack of RNAIII expression, low expression levels of the positively regulated extracellular factors such as hemolysins, proteases, and TSST-1, and high expression levels of the negatively regulated factors, such as Spa. It has thus been speculated that the KD isolates may represent a new clone of TSST-1-secreting S. aureus, with a partially defective agr locus, or that isolates may be bona fide agr-defective mutants which have reverted in their ability to express TSST-1 (34).

In this study, we further characterized the KD isolates by examining the level of Spa expressed by these isolates compared to TSS isolates. We report that, when considered as a group, the KD isolates express significantly higher levels of cell wall-associated and extracellular Spa than TSS isolates, another characteristic of an agr-defective phenotype. However, consistent with previous observations, no difference was seen in the level of TSST-1 expressed by the two groups of isolates. We investigated the possibility that the KD isolates may be partially defective in their agr loci by analyzing spa and RNAIII transcription levels and by nucleotide sequence analysis of the RNAIII-coding region.

MATERIALS AND METHODS

Bacterial strains and growth conditions. S. aureus strains Cowan I and Wood 46 were obtained from the American Type Culture Collection (Rockville, Md.). S. aureus strains 8325-4, RN4228, and RN4256 (RN4228 agr4; Tn551) were kindly provided by R. P. Novick (Public Health Research Institute, New York, N.Y.). TSST-1-expressing S. aureus isolates were obtained from either pharyngeal or rectal swabs of 21 patients with acute KD. Each of these patients fulfilled the diagnostic guidelines of the American Heart Association (3). TSST-1-expressing vaginal isolates of S. aureus were obtained from 12 patients with menstrual TSS. S. aureus strains were grown in tryptic soy broth (TSB). Escherichia coli JM101 (51) was used for plasmid DNA transformations and was grown in Luria-Bertani broth, containing 100 μg of ampicillin per ml when appropriate.

Analysis of cell wall-associated Spa expression by flow cytometry. Overnight cultures were diluted 1:50 in 5 ml of fresh TSB and were grown at 37°C to early and late exponential phases (Fig. 1A, t1 and t2, respectively). All the isolates had similar growth rates and equivalent OD600 values at each 1-h time point. Cells were pelleted at 2,500 × g for 10 min at 4°C and then resuspended and blocked in staining solution (5% fetal calf serum in 4% paraformaldehyde in 1% sodium azide in PBS) and finally resuspended in PBS. Stained cells were fixed with 3% methanol-1% paraformaldehyde for 30 min at 4°C. Cells were then washed twice in a solution containing 1% bovine serum albumin (BSA) in PBS for 2 h at 4°C and the pelleted at 2,500 × g for 10 min at 4°C. The cells were then washed twice with PBS/T, 100 μl of 1% BSA in PBS/T per well for 2 h at room temperature. After washing twice with PBS/T, 100 μl of each supernatant was added to the wells, and the plate was incubated for 2 h at 37°C. The plate was washed three times with PBS/T and then incubated with horseradish peroxidase-conjugated anti-TSST-1 antibodies (Toxin Technology) for 1 h at 37°C. Wells were washed 10 times with PBS/T, 100 μl of Avidin-Biotin complex (ABC) conjugated with horseradish peroxidase (Sigma) was added to each well, and the plate was incubated at room temperature for 15 min. After washing twice with PBS/T, 100 μl of each diluted supernatant sample was added to the wells, and the plate was incubated for 2 h at 37°C. The plate was washed three times with PBS/T and then incubated with horseradish peroxidase-conjugated anti-SpA MAb (Sigma) for 1 h at 37°C. Wells were washed 10 times with PBS/T, 100 μl of SPA conjugated with biotin (Sigma) was added to each well, and the plate was incubated at room temperature. After 15 min, the reaction was quenched by adding 50 μl of stop solution to each well. OD405 was determined. A standard curve was prepared with known concentrations of the purified Staphylococcus aureus TSS-1 antigen (Toxin Technology, Sarasota, Fla.) per ml of 0.1 M NaHCO3 buffer (pH 8.2) at 37°C. The plate was then washed twice with PBS/T and blocked with PBS/T for 30 min at room temperature. After washing twice with PBS/T, 100 μl of diluted supernatant sample was added to the wells, and the plate was incubated for 2 h at 37°C. The plate was washed three times with PBS/T and then incubated with horseradish peroxidase-conjugated anti-TSST-1 antibodies (Toxin Technology) for 1 h at 37°C. Wells were washed 10 times with PBS/T, 100 μl of Avidin-Biotin complex (ABC) conjugated with horseradish peroxidase (Sigma) was added to each well, and the plate was incubated at room temperature. After 15 min, the reaction was quenched by adding 50 μl of stop solution to each well. OD405 was determined. A standard curve was prepared with known concentrations of the purified TSST-1 (Toxin Technology).

Isolation of RNA and Northern hybridization analysis. Total cellular RNA was isolated from cultures grown to early and late exponential phases (Fig. 1A, t1 and t2, respectively) by using the Insergan Mini Kit (Qiagen, Valencia, Calif.). Each of the isolates had similar growth rates and equivalent OD600 values at each time point. Ten micrograms of each RNA sample (as determined by measuring the OD260) was resolved by 1.2% agarose-formaldehyde gel electrophoresis. RNA markers (Ambion, Austin, Tex.) were used to size standards. RNA was transferred onto a Hybond N+ membrane (Amer sham Life Science, Inc., Arlington Heights, Ill.) and was hybridized with radioactively labeled DNA probes at 42°C, as described by Sambrook et al. (45). Probes were radiolabeled by nick-translation with [α-32P]dCTP and were dot-blotted on a Hybond N+ membrane (Ambion). A 945-bp fragment (nt 581 to 1526) of the spa gene was used to delineate the TSST-1-expressing isolates. The probe was radiolabeled with [α-32P]dCTP and was hybridized to the RNA samples at 42°C. A specific signal was detected at 104 copies per μg by using the Radiolabel DNA Labeling System ( Gibco BRL, Grand Island, N.Y.). Probe DNA for the spa and RNAIII transcripts was amplified by PCR from the chromosomes of strains Cowan I and 8325-4, respectively, as follows: spa, 1.346-kb fragment (nucleotide [nt] 771 to 2117, according to Shutte-oldworth et al. [46]) by using the primers 5′-TTATATCTGGTGGCCGTAACACC TGTC-3′ and 5′-ATCCTGTAGGCTTTGACGATC-3′, and RNAIII, a 945-bp fragment (nt 581 to 1526, according to Jän zer and van der Auwera et al. [21]) containing the entire RNAIII-coding sequence and the 5′ half of aggr by using the primers 5′-AAATAACATGACTGACCTGTCAGGG-3′ and 5′-TTATGAATGTCGCA GGAATGG-3′. After hybridization, membranes were washed twice in a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS at 65°C and once in 0.1× SSC-0.1% SDS at 65°C, for 30 min each time. Membranes were exposed to Kodak X-Omat AR film at −80°C for various lengths of time.

Molecular cloning and sequencing. An 862-bp fragment containing the entire RNAIII-coding region and approx. 1175 bp of upstream sequence (nt 314 to 1175, according to Jän zer and van der Auwera et al. [21]) was amplified by PCR from the chromosomes of strain 8325-4, five KD isolates (Yeh, KD1, KD6, KD-ATK, and 1175), according to Jän zer et al. [21]) containing the entire RNAIII-coding sequence and the 5′ half of aggr by using the primers 5′-AACATGACTGACCTGTCAGGG-3′ and 5′-TTATGAATGTCGCA GGAATGG-3′. After hybridization, membranes were washed twice in a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS at 65°C and once in 0.1× SSC-0.1% SDS at 65°C, for 30 min each time. Membranes were exposed to Kodak X-Omat AR film at −80°C for various lengths of time.

RESULTS

Determination of cell wall-associated Spa expression levels. The levels of cell wall-associated Spa produced by early- and late-exponential-phase (Fig. 1A, t1 and t2, respectively) cultures of the KD and TSS isolates were compared by flow cytometry, using a biotinylated anti-Spa MAb and phycoerythrin-conjugated streptavidin. The MFI observed gave a relative measure of the cell wall-associated Spa expressed by each isolate (Fig. 2). Strains Cowan I and Wood 46 were...
included in this study as examples of strains which express high and very low levels of SpA, respectively. Strain RN4256 was also included as an example of an agr mutant that lacks RNAIII, a negative regulator of spa gene transcription. As anticipated, strains Cowan I and RN4256 produced significantly higher levels of cell wall-associated SpA than strain Wood 46 at both growth phases analyzed. When compared collectively, the KD isolates (n = 14) expressed significantly higher levels of cell wall-associated SpA than the TSS isolates (n = 11) at the early exponential growth phase (MFI of 950.04 ± 349.38 versus 95.98 ± 58.79; P < 0.05, Student’s t test). The same result was observed for late-exponential-phase cultures of the KD and TSS isolates (MFI of 1,597.07 ± 368.70 versus 228.99 ± 134.90; P < 0.05, Student’s t test).

Production of extracellular SpA. The levels of extracellular SpA secreted by early- and late-exponential-phase cultures (Fig. 1A, t1 and t2, respectively) of the KD and TSS isolates were determined by ELISA (Fig. 3). Again, strains Cowan I, Wood 46, and RN4256 were included in the study as controls. When compared collectively, the KD isolates (n = 21) were found to secrete significantly higher levels of SpA than the TSS isolates (n = 12) at both growth phases analyzed (0.54 ± 0.10 versus 0.28 ± 0.22 μg/ml for early-exponential-phase cultures and 1.22 ± 0.42 versus 0.51 ± 0.35 μg/ml for late-exponential-phase cultures; P < 0.05, Student’s t test). However, when considered individually, a few TSS isolates were observed to express high levels of extracellular SpA comparable to the KD isolates.

Production of TSST-1. The amounts of TSST-1 secreted by late-exponential-phase cultures (Fig. 1A, t2) of the KD (n = 19) and TSS (n = 12) isolates were measured by ELISA (Fig. 4). The level of TSST-1 secretion was highly variable among...
individual isolates, ranging from 0.25 to 30.0 ng/ml. Furthermore, in contrast to cell wall-associated or extracellular SpA, there was no statistically significant difference in the levels of TSST-1 secreted by the KD or TSS groups of isolates at this growth phase (8.55 ± 5.41 and 10.00 ± 9.39 ng/ml, respectively).

Analysis of spa and RNAIII transcript levels. To investigate the possibility that KD isolates may have a partially defective agr locus, the levels of spa and RNAIII transcripts in early- and late-exponential-phase cultures (Fig. 1A, t1 and t2, respectively) of the KD (n = 9) and TSS (n = 11) isolates were compared by Northern hybridization analysis. Strains Cowan I, Wood 46, and RN4282 and its isogenic, agr-negative mutant, RN4256, were included in the study as controls. The results obtained for some of the isolates analyzed are shown in Fig. 1B and 1C. High levels of spa mRNA (ca. 1.8 kb) were detected at both growth phases in all the KD isolates analyzed (Fig. 1B, lanes 3 to 7, and data not shown), strain RN4256 (Fig. 1B, lane 2), and strain Cowan I (Fig. 1B, lane 12). This is consistent with the finding that these strains express high levels of SpA. A similar result was observed for a few TSS isolates (Fig. 1B, lanes 10 and 11, and data not shown). These TSS isolates expressed high levels of SpA, comparable to the KD isolates. The spa mRNA was undetectable or barely detectable in the remaining TSS isolates analyzed (Fig. 1B, lanes 8 and 9, and data not shown), strain RN4282 (Fig. 1B, lane 1), and strain Wood 46 (Fig. 1B, lane 13). This is consistent with the observation that SpA is expressed at significantly lower levels by these strains.

The RNAIII transcript (ca. 510 bp) was detected in both early- and late-exponential-phase cultures of all the strains analyzed, except for strains RN4256 and Cowan I (Fig. 1C and data not shown). This is consistent with strain RN4256 being an agr-defective mutant. It also suggests that Cowan I has an uncharacterized agr defect, which may explain the high level of SpA expressed by this strain. The RNAIII transcript was more abundant in the late-exponential-phase cultures of all the isolates tested, consistent with previous reports on the regulation of the expression of this factor. However, significantly less RNAIII was detected in the early-exponential-phase cultures of all the KD isolates and a few of the TSS isolates than of strains RN4282, Wood 46, and the remaining TSS isolates. Furthermore, an inverse correlation was observed between the levels of the RNAIII and spa transcripts detected for each strain at this growth phase. This is consistent with RNAIII functioning as a negative regulator of spa gene transcription. Interestingly, despite the elevated levels of RNAIII in the late-exponential-phase cultures of the KD isolates and certain TSS isolates, the amount of spa mRNA did not decrease dramatically in these isolates.

DNA sequence analysis of the RNAIII-coding region. Although all the KD isolates analyzed express RNAIII, it is possible that this transcript may not be functional in these strains. To address this question, the DNA sequence of a ca. 860-bp chromosomal fragment encompassing the RNAIII reading frame, the RNAIII P3 promoter, and the agr operon P2 promoter of five KD isolates and strains MN8 and FRI 1169 (TSS isolates) was determined. Each sequence was identical except for a single nucleotide substitution 2 nt upstream from the RNAIII transcriptional start site. The sequences of the other

![Graph](http://iai.asm.org/Graph.png)

**Fig. 2.** Cell wall-associated SpA expression by KD and TSS isolates of *S. aureus*. Relative expression of SpA was determined using flow cytometry by measuring the MFI after incubation with biotinylated anti-SpA MAb and phycoerythrin-conjugated streptavidin, as described in the text. Each value represents the mean of duplicate analyses on the same isolate during the early and late exponential growth phases (t1 and t2 in Fig. 1A, respectively). Strains RN4256, Cowan I, and Wood 46 were included in the study as controls. KD isolates were found to express significantly (P < 0.05) more SpA on their cell surface than TSS isolates as calculated by Student’s t test.
strains differed from that of strain 8325-4 in 10 nucleotide positions. However, all of these substitutions were outside the RNAIII-coding region and the -10 and -35 boxes of both the P2 and P3 promoters. There were also two insertions in the MN8/KD isolate sequence compared to strain 8325-4. A single nucleotide insertion which was not predicted to affect the RNAIII secondary structure was found at the 3' end of the RNAIII-coding region (but outside the hld gene), and a 4-nt insertion was found just downstream from the RNAIII transcriptional termination site. Therefore, the sequence of the RNAIII molecule appears to be highly conserved in all the strains analyzed, with no apparent mutations that might affect the functionality or transcription level of the molecule.

DISCUSSION

It has been previously reported that TSST-1-secreting strains of *S. aureus* isolated from patients with KD exhibit an unusual phenotype which distinguishes them from TSS isolates, including the expression of lower levels of hemolysins, lipase, and protease (34). In this study, we describe another phenotype associated with KD isolates. When considered collectively, these isolates were found to express significantly more cell wall-associated and extracellular SpA than isolates from patients with TSS. This correlated with a higher level of *spa* mRNA expression in the KD isolates. Nonetheless, when considered individually, a few TSS isolates which expressed levels of *spa* mRNA and SpA comparable to the KD isolates were observed. Thus, high levels of SpA expression are not unique to the KD isolates but do appear to be a characteristic phenotype of these strains. In contrast, the KD and TSS isolates expressed comparable levels of TSST-1 in this study, consistent with previous findings (34).

It was recently suggested that the KD isolates might have a defective *agr* regulatory system that may be responsible for the usual phenotype associated with these strains (34). The high levels of SpA expressed by the KD isolates in this study are also typical of *agr*-defective mutants. Interestingly, the *agr* locus is genetically unstable in vitro (4, 37). Therefore, in this study, we chose to investigate the possibility that the KD isolates may have an *agr* defect. The *agr* locus consists of two divergent transcripts, RNAII, which contains *agrB, -D, -C*, and -A, and RNAIII, the effector molecule of the regulatory system (20, 41). The transcription of RNAIII is regulated by the *agr* system itself, and RNAIII is not transcribed in mutants that have a defect in any of the *agr* genes (40). In this study, all of the isolates analyzed expressed RNAIII, suggesting that all of the *agr* genes are functional. In addition, nucleotide sequence analysis of the RNAIII-coding regions of five representative KD isolates suggested that the RNAIII molecules were not mutated in these strains. Taken together, these results suggest that the KD isolates are not bona fide *agr*-defective mutants.

In this study, a correlation was observed between the levels of RNAIII and *spa* mRNA detected in each isolate in the early exponential phase (Fig. 1B and 1C, **t.**) In the TSS isolates which produced low levels of *spa* mRNA and SpA protein, high levels of RNAIII were detected (Fig. 1B and 1C, lanes 8 and 9, and data not shown). This suggests that the high levels of RNAIII in these isolates may be suppressing the transcription of the *spa* mRNA. In contrast, high levels of *spa* mRNA were detected in early-exponential-phase cultures of the KD isolates and in the few TSS isolates that expressed high levels of SpA.
indirectly suppresses spa mRNA transcription. How the sar locus suppresses spa mRNA transcription independently of agr has not yet been determined. Given that in sarA-defective mutants protease expression is increased and RNAIII transcription is greatly reduced or absent (5, 8, 10, 19), it seems unlikely that the KD isolates are defective in the sar regulatory locus.

It is clear from this and previous studies that the KD isolates have similar phenotypes (34). In addition, our preliminary observations suggest that these isolates may also be very similar at the genotypic level. We have noted that the chromosomal restriction enzyme digestion profiles of these isolates are very similar, if not identical. Furthermore, Southern hybridization and PCR analyses of the spa and coagulase loci of these strains suggest that they are highly conserved, encoding five N-terminal IgG-binding repeats and four C-terminal fibrinogen-binding repeats, respectively (unpublished data). In addition, the nucleotide sequence of a ca. 860-bp chromosomal region encompassing the RNAIII-coding region and flanking DNA was identical in the KD isolates analyzed. Indeed, it is tempting to speculate that the KD isolates may be clonal in origin, as is the case for TSS isolates (39). The KD isolates used in this study were collected from a variety of geographical locations within the United States. A multicenter epidemiological study of KD isolates using traditional typing methods, such as restriction fragment length polymorphism and multilocus enzyme electrophoresis analysis, or newer typing methods based on DNA sequence analysis is warranted to further investigate the clonality of these isolates.

It is a tantalizing possibility that the expression of high levels of extracellular SpA, secreted locally by S. aureus isolates colonizing the gastrointestinal tract of patients, may contribute to the symptoms of KD. It has been recently demonstrated that, in addition to exhibiting immunoregulatory activities, SpA has the ability to bind to von Willebrand factor, a protein that has a central role in hemostasis and thrombogenesis (18). This raises the exciting possibility that, in addition to inducing immune activation, SpA could perturb host hemostasis and increase the risk of vascular thrombosis, such as that associated with KD. Interestingly, it has been noted that coronary complications develop in some patients diagnosed with TSS (12, 49). Whether these patients are infected with S. aureus strains that secrete high levels of SpA remains to be determined. Significantly, such TSS isolates were observed in this study. However, additional bacterial and host factors are likely to be involved in determining whether a patient develops classical TSS or KD (35). Investigation of the potential role of SpA in the development of KD will require an animal model of this disease in which the contribution of this and other factors can be analyzed.

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