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Autoinducer of Virulence As a Target for Vaccine and Therapy Against *Staphylococcus aureus*

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Staphylococcus aureus causes pathologies ranging from minor skin infections to life-threatening diseases. Pathogenic effects are largely due to production of bacterial toxin, which is regulated by an RNA molecule, RNAIII. The *S. aureus* protein called RAP (RNAIII activating protein) activates RNAIII, and a peptide called RIP (RNAIII inhibiting peptide), produced by a nonpathogenic bacteria, inhibits RNAIII. Mice vaccinated with RAP or treated with purified or synthetic RIP were protected from *S. aureus* pathology. Thus, these two molecules may provide useful approaches for the prevention and treatment of diseases caused by *S. aureus*.

Staphylococcus aureus is a Gram-positive bacterial pathogen that causes diseases ranging from minor skin infections to life-threatening deep infections such as pneumonia, endocarditis, meningitis, postoperative wound infections, septicemia, and toxic shock syndrome (*1*). Hospitalized patients are at particular risk; over 500,000 nosocomial infections are reported per year (*2*). The emergence of drug resistance has made many of the available antimicrobial agents ineffective. Therefore, alternative methods for prevention and treatment of bacterial infections in general and of *S. aureus* infections in particular are eagerly sought.

Like other Gram-positive bacteria, *S. aureus* causes diseases chiefly by production of virulence factors such as hemolysins, enterotoxins, and toxic shock syndrome toxin. The synthesis of virulence factors in *S. aureus* is controlled by a regulatory RNA molecule, RNAIII (*3–5*), encoded by the *agr* locus. The *rnaiii* gene is transcribed in culture only from the mid-exponential phase of growth and is autoinduced by the RNAIII activating protein (RAP) (*6*). RAP is continuously secreted by the bacteria and activates *rnaiii* only at a concentration threshold (*6*). Antibodies to RAP block the activation of *rnaiii* in vitro. A peptide, termed RNAIII inhibiting peptide (RIP), is produced by a nonpathogenic strain of *S. aureus* mutated by nitrosoguanidine (*6*). RIP competes with RAP for activation of *rnaiii* and thus inhibits production of toxin by *S. aureus* (*6*). This suggests that interference with activation of the *agr* system by blocking the autoinducer RAP or by supplying RIP may inhibit the expression of all toxins regulated by *rnaiii*, thereby interfering with pathogenesis. We report that mice vaccinated with RAP or treated with RIP are protected from *S. aureus* infection.

We purified RAP from postexponential supernatants of wild-type *S. aureus* (Fig. 1) as described (*6*) and the peak fraction (Fig. 1, B and C) contained a protein of about 38 kD (Fig. 1D). We also purified RAP from postexponential supernatants of an *agr*-null strain in which the entire *agr* locus was replaced with the *tetM* marker (*7*) (Fig. 1, B and D), which suggests that RAP is independent of *agr*.

To test whether immunization with RAP can inhibit *S. aureus* infection, we used the murine model of cutaneous infection (*8*). When the wild-type Smith diffuse (SD) strain of *S. aureus* is injected subcutaneously together with dextran beads (Cytodex), a visible, measurable lesion (cellulitis) is induced after 24 hours. In contrast, no lesion is induced in animals injected with Cytodex beads alone (*9*).

Mice were vaccinated (*10*) with RAP purified (*6*) from an *agr*-null strain (RAP*) or from a wild-type *S. aureus* strain (RAP) (*9*) (Table 1). After they were challenged with *S. aureus*, 72% (24 of 33) of RAP-vaccinated mice remained free of disease compared with 30% (3 of 10) of control mice immunized with complete Freund's adjuvant (CFA) and none of the untreated controls. This difference was statistically significant (Fisher's exact

Table 1. Vaccination or suppression of *S. aureus* SD infections (*10, 16*).

Treatment group	No. of mice	No lesions		Lesions		Death	
		n	(%)	*n*	Mean size (mm^2)	*n*	(%)
Vaccination with RAP as an antigen							
RAP	24	17	(71)	6	96	1	(4)
RAP*	9	7	(78)	2	84	0	(0)
CFA	10	3	(30)	5	177	2	(20)
Untreated	12	0	(0)	9	370	3	(25)
RIP suppression of 8.5 × 10^7 SD							
SD+RIP	4	3	(75)	1	33	0	(0)
SD+saline	4	1	(25)	3	39	0	(0)
RIP suppression of 1.4 × 10^8 SD							
SD+RIP	8	4	(50)	4	45	0	(0)
SD+saline	6	0	(0)	6	100	0	(0)
RIP and Pep suppression of 1.4 × 10^9 SD							
SD+RIP	10	3	(30)	3	39	4	(40)
SD+saline	10	2	(20)	6	160	2	(20)
SD+Pep	10	9	(90)	1	56	0	(0)
SD+DMSO	9	2	(20)	4	128	3	(22)

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probability test: RAP versus untreated, $P < 0.0001$; RAP versus CFA, $P = 0.0031$) (*11*). In addition, among mice that developed lesions, the mean lesion size (*12*) for RAP-vaccinated mice (90 mm^2) was 50% smaller than in CFA control mice (177 mm^2) and 76% smaller than in untreated controls (370 mm^2). Animals that died had extensive lesions that spread to over one-quarter of their body. Only 3% (1 of 33) of RAP-vaccinated animals died as a result of challenge, whereas 22% (5 of 22) of control animals died.

To determine whether antibodies to RAP were generated, sera from vaccinated and control animals were analyzed by immunoblotting and binding activity to wild-type *S. aureus* postexponential supernatants that contained RAP (*13*, *14*) (Fig. 2A). Animals vaccinated with RAP developed antibodies to a 38-kD protein and could also bind to purified RAP.

Most (33 of 34) vaccinated animals developed antibodies to RAP (titers ranged from 1:50 to 1:16,000) as a result of the injections; none of the control animals developed antibodies. However, some mice had preimmune antibodies to RAP (titers ranged from 1:40 to 1:2000), and they developed smaller lesions when challenged with *S. aureus*. The titer of antibodies to RAP (in 81 vaccinated or control animals) inversely correlated with lesion size (Fig. 2B). For the purposes of this calculation, animals that died (of extensive cellulitis) were assumed to have a lesion size of 981 mm^2 (*11*).

A nonpathogenic *S. aureus* produces the peptide RIP, which inhibits *rnaiii* transcription (Fig. 3) and competes with RAP for activation of virulence (*6*). The high-performance liquid chromatography (HPLC)–purified RIP (*6*) was subjected to Edman degradation sequencing. The amino acid sequence was determined to be YSPXTNF (*15*). A synthetic peptide corresponding to YSPWTNF (Pep) was synthesized and tested for its ability to inhibit *rnaiii* in vitro. The synthetic peptide (Pep) inhibits induction of *rnaiii* comparably to RIP (Fig. 3).

We tested purified (*6*) and synthetic RIP for the ability to suppress infection in the murine cutaneous *S. aureus* infection model (*16*). For these experiments, we prescreened mice to eliminate individuals with antibodies to RAP. A fixed amount of RIP (about 10 μg) attenuated infections caused by increasing inocula of the SD strain of *S. aureus*. Of the animals that were injected with 8.5×10^7 bacteria together with RIP, three of four developed no infection at all, compared with only one of four control animals that were injected with the bacteria and saline (Table 1). When we used an increased inoculum of bacteria (1.4×10^8 cells per injection), four of eight animals were protected, and the remaining four developed a lesion that was 55% smaller than in control animals

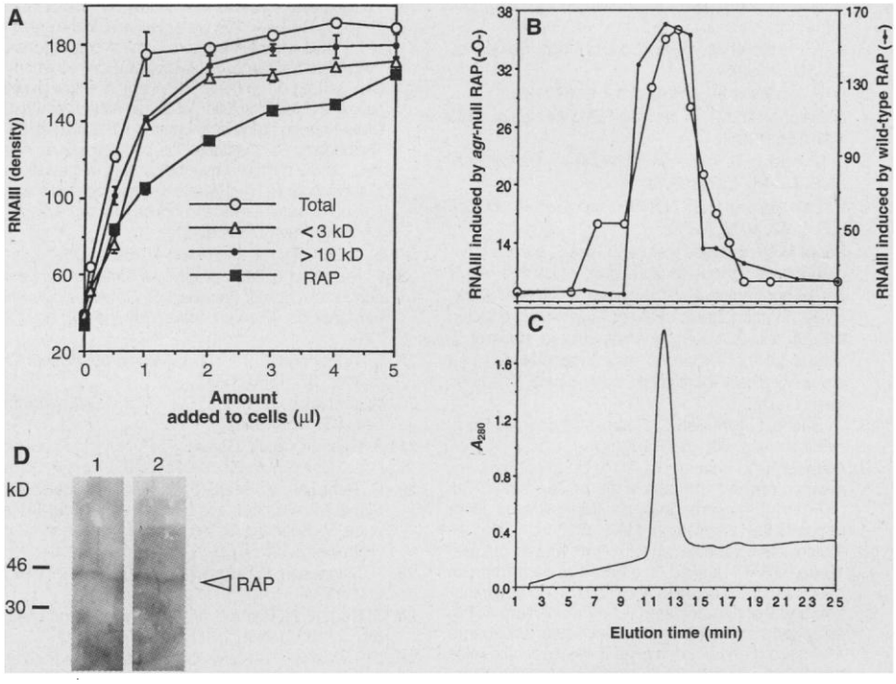

Fig. 1. Purification of RAP. (**A**) Postexponential supernatants of wild-type *S. aureus* (total) were filtered through a 3-kD cutoff membrane. Flowthrough containing *agrD*-encoded octapeptide (*21*) (<3 kD) was tested for *rnaiii* activation as described (*6*). Retained material (containing RAP) was filtered through a 10-kD cutoff membrane. Material greater than 10 kD (>10 kD) was applied to an HPLC gel-filtration column and purified RAP was collected (RAP). Increasing amounts of material at each purification step were added to early exponential wild-type *S. aureus* and tested for the ability to activate *rnaiii* (*6*). (**B**) Postexponential supernatants of wild-type *S. aureus* and of *agr*-null *S. aureus* were fractionated on a gel-filtration column at 1 ml/min (*6*). Each 1-ml fraction was collected and tested for its ability to activate *rnaiii* (*6*). (**C**) Absorbance of fraction containing peak *rnaiii*-inducing activity. (**D**) Gel-filtration purified RAP from wild type (lane 1) and from *agr*-null strain (lane 2) was separated on SDS–polyacrylamide gel and silver stained. Approximate molecular mass markers are indicated.

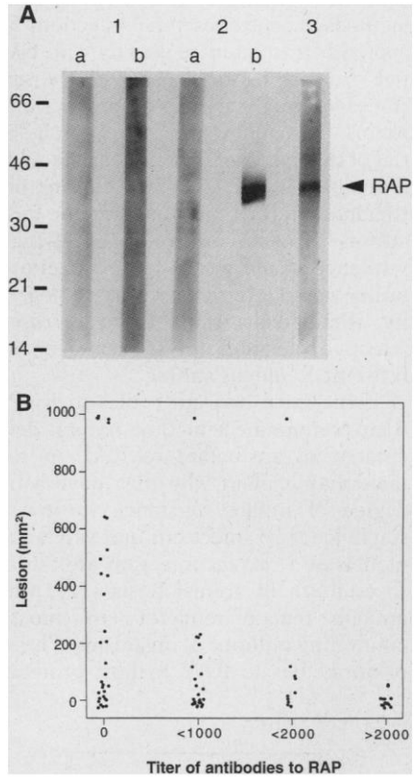

Fig. 2. Immunoblotting of sera of vaccinated and control animals. Postexponential supernatant of wild-type *S. aureus* (lanes 1 and 2) or purified RAP (lane 3) was separated on 12% SDS-polyacrylamide gel and Western blotted, and membranes were incubated in the presence of preimmune (lane 1a) or postimmune (lane 1b) serum collected from a control CFA-injected animal (diluted 1:20). Lane 2, preimmune (lane 2a, diluted 1:20) or postimmune (lane 2b, diluted 1:1000; lane 3, diluted 1:20) serum. Approximate molecular size markers are indicated on the left. (**B**) Titer of RAP antibodies versus lesion size. The titer of RAP antibodies from vaccinated and control animals was determined by Western blotting against postexponential supernatants containing RAP. Lesion size of animals challenged with *S. aureus* was determined. The largest lesions were in animals that died of extensive cellulitis (about 981 mm^2).

(Table 1). All the control animals (seven of seven) challenged with SD and saline developed a lesion. When more bacteria were used (1.4×10^9), the synthetic RIP (0.5 mg of Pep) protected animals—90% (9 of 10) of the animals showed no sign of disease (Table 1). These results suggest that the ratio between RIP and the bacteria is critical and helps determine the success of the host's immune response to eliminate the bacteria.

Current bacterial vaccines target *S. aureus* or the exotoxins it produces (*17*, *18*), but these approaches met with limited success. With an inexorable increase in antibiotic resistance among bacteria in general (*19*) and among staphylococci in particular (*20*), there is a need to develop innovative methods to control bacterial infections. Our approach is to interfere directly with bacterial virulence by interfering with the signal transduction that leads to production of toxins. By reducing the pathogenic potential of the bacteria, this approach would be synergistic with current antimicrobial therapies and natural host immune mechanisms. Because directed suppression of virulence would not kill the bacteria but rather would interfere with its pathogenicity, there likely would be a decrease in selective pressures for the emergence of resistant *S. aureus* strains.

Some mice had antibodies to RAP in their preimmune sera. The natural development of antibodies to RAP in some mice may explain why they have a high degree of innate resistance to invasive staphylococcal infections and why animal models of *S. aureus* infection are difficult to establish in animal hosts (*17*), where animals remain refractory to infection containing millions of organisms. The role of antibodies to RAP in host protection remains an important question.

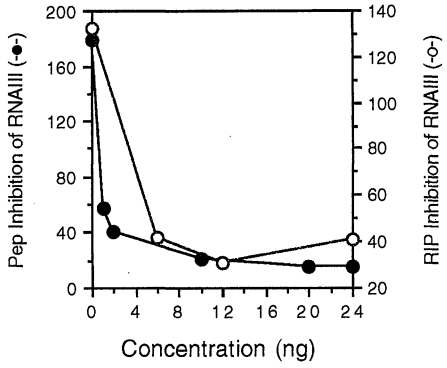

Fig. 3. Inhibition of RNAIII by native and synthetic RIP. Increasing amounts of RIP, which was purified on a C_{18} column (*6*), or increasing amounts of synthetic peptide (Pep) were added to early exponential wild-type *S. aureus* and tested for the ability to inhibit *rnaiii* (*6*). Density of RNAIII is shown.

Animals vaccinated with RAP that was purified from a *S. aureus agr*-null strain (*7*) had the same degree of protection as animals vaccinated with RAP that was purified from a wild-type strain. This rules out contribution of products from other genes known to regulate RNAIII, such as the octapeptide encoded by *agrD* (*21*).

Regulatory mechanisms involving autoinducers have been described for other bacterial systems (*22*), including competence and sporulation in *Bacillus subtilis* and in *Streptococcus pneumoniae* (*23*), conjugation in *Enterococcus faecalis* (*24*), and elastase production in *Pseudomonas aeruginosa* (*25*). Furthermore, compounds have been identified that inhibit phosphorylation of the bacterial two-component signal transduction system in *P. aeruginosa* (*26*). Targeting the autoinducers of virulence or the signal transduction they activate therefore may be useful in preventing pathogenesis of other bacteria known to be regulated by global regulons.

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7. Bacterial strains used were wild-type *S. aureus*; nonpathogenic *agr*-null *S. aureus* in which the whole 3.4-kb *agr* fragment was replaced by the *tetM* marker (*3*); nonpathogenic strain of *S. aureus* producing RIP (*6*); and SD, a highly encapsulated strain of *S. aureus* (*8*) that produces exopolysaccharides that are antigenically identical to many clinical *S. aureus* strains tested (*27*).
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9. Animals [4- to 8-week-old (20 to 30 g) outbred, immunocompetent hairless male mice, strain Crl: SKH1(hrhr)Br] were obtained from Charles River Laboratories (Wilmington, MA).
10. Prophylactic vaccination with RAP was as follows: 10 μg of RAP, purified on a gel-filtration column as described (*6*), was injected with CFA on first injection and with incomplete Freund's adjuvant (ICFA) on second and third injections subcutaneously into 4-week-old immunocompetent hairless male mice on days 0, 7, and 21. Control mice were either injected with the adjuvant alone or not injected at all (untreated). Vaccinated and control mice were challenged on day 31 with 1.24×10^8 SD *S. aureus* subcutaneously together with 1 mg of Cytodex beads (*10*) to induce a local infection. The size of the lesion was measured daily (*14*). Of note is that animals injected only with Cytodex beads developed no lesions at all.
11. Fisher's exact probability test was used to compare proportions of mice developing lesions and mice developing RAP antibodies among the experimental groups (RAP vaccinated, CFA controls, untreated controls). Among animals that developed lesions after challenge with *S. aureus*, the size of the lesions was compared by single-factor analysis of variance. Post-hoc testing was done by Fisher's protected least significant difference.
12. Lesions were measured 24 to 48 hours after challenge and are presented as area = $0.5[\pi(\text{length}) \times (\text{width})]$.
13. To estimate amount of antibody to the injected antigen, we collected a drop of blood (50 μl) from the tip of the tail before vaccination (preimmune sera) and 7 days after the third vaccination period (postimmune sera, 3 days before bacterial challenge). RAP antibody titer was determined by Western blotting. Serum was added to the blot (containing postexponential supernatants) in increasing dilutions until no band appeared. The highest dilution that still reacted with RAP is the titer determined.
14. For Western blotting, 30 μl of wild-type *S. aureus* (concentrated 10:1) postexponential supernatant or 5 μg of purified RAP was separated on a 12% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane; the membrane was blocked for 1 hour in 3% bovine serum albumin (BSA) in tris-buffered saline (TBS). The membrane was then incubated with the serum (diluted in 1% BSA/TBS) for 2 hours at 22°C, washed three times for 10 min in TBS containing 0.05% Tween 20 (TBST), and incubated in peroxidase-conjugated mouse immunoglobulin G (Boehringer) for 1 hour at 37°C in TBST. Bound antibody was detected by chemiluminescence with the ECL Western blotting system (Amersham).
15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; U, Tyr; X, unknown or other.
16. To suppress *S. aureus* infection by RIP, SD *S. aureus* (8.5×10^7 to 1.4×10^9) were incubated in the presence of RIP that was purified from 5 ml of postexponential culture broth in saline (*6*), or with saline only as a control, with 0.5 mg of synthetic RIP (Pep) in a final dimethyl sulfoxide (DMSO) solution of 3% (in saline), or with 3% DMSO only in saline as a control, for 30 min at 37°C. The bacteria-RIP, bacteria-Pep, bacteria-saline, or bacteria-DMSO mixture was injected subcutaneously with Cytodex beads (1 mg) (*8*) into 8-week-old immunocompetent hairless male mice to induce a local infection. The lesion was measured daily (*12*).
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