JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1977, p. 233-237 Vol. 6, No. 3
Printed in U.S.A.
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Teichoic Acids in Pathogenic *Staphylococcus aureus*

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Received for publication 12 April 1977

Twenty-six strains of *Staphylococcus aureus* obtained from patients with endocarditis were studied for the production of alpha- and/or beta-ribitol teichoic acid (TA), using highly specific anti-TA antibodies prepared in rabbits. A counterimmunoelectrophoretic assay was used. Beta-TA was the predominant residue produced by all strains; alpha-TA was found in all strains, but in smaller amounts and with much strain-to-strain variation. Antibodies in patients' sera were found against beta-TA in higher titers and for longer periods than were anti-alpha-TA antibodies. Antibodies against one or both TA residues were present in all but one of 26 patients.

The cell walls of coagulase-positive *Staphylococcus aureus* contain a ribitol teichoic acid (TA) with either alpha- or beta-linked *N*-acetylglucosamine residues (1, 10, 15). Ribitol TA may be associated with pathogenicity of strains of staphylococci isolated from human infections: 97 of 100 strains of *S. aureus* isolated from human infections contained ribitol TA in their cell walls (5). Furthermore, Oeding (12) reported that of serological analysis of 24 strains of *S. aureus* from human infections, 67% contained the alpha residue and 96% contained the beta residue. These results agree with previous chemical analysis of staphylococcal cell wall TAs which indicated that most human pathogenic staphylococci contain both the alpha and the beta forms of ribitol TA (15). The examination of extracellular TAs in culture broth, although not as accurate as chemical analysis of the disrupted cell walls, has been widely used to investigate the distribution of the polysaccharide among strains isolated from animals (12, 13) and is felt to reliably reflect intracellular production and distribution of TAs.

In recent years, serological methods have been developed for the detection of TA antibodies in patients with staphylococcal endocarditis (3, 11). In these studies a crude mixture of alpha and beta residues of ribitol TA derived from sonically treated cell walls of the Lafferty strain of *S. aureus* was used; this strain contains both residues (11). The distribution of these two antigens and the incidence of specific antibody response to each residue in a well-defined human infection with *S. aureus* has not been clarified. We have therefore examined 26 strains of *S. aureus* obtained from parenteral drug abusers with staphylococcal endocarditis and have determined the relative content of alpha- and beta-ribitol TAs. In addition, the specific antibody response to both residues was determined for each case and followed throughout the course of the disease.

MATERIALS AND METHODS

Organisms. Twenty-six strains of coagulase-positive *S. aureus* were isolated from blood cultures of parenteral drug users with staphylococcal endocarditis. Clinical and microbiological descriptions of some of these cases have been published (16). Two additional human strains of known antigenic composition served as controls: *S. aureus* strain 3528 contained predominantly alpha-*N*-acetylglucosamine TA in the cell wall, whereas strain 845 contained beta-linked residues. (Both strains were kindly provided by G. W. Ross, Glaxo Laboratory, Greenford, England.) The chemical analyses of these two strains have been completely detailed (10). All strains were maintained on Trypticase soy agar. Control strains from non-drug users were not included, since the phage types as well as antibiotic sensitivity patterns of these strains were not different from those of strains obtained from community controls (16).

Before the organisms were assayed for antigenic composition, an overnight culture of each strain in Trypticase soy broth was prepared. The overnight cell suspension was used in the counterimmunoelectrophoretic (CIE) assay without further purification.

Immunization of rabbits. Antisera against *S. aureus* strains 3528 and 845 were induced by weekly intravenous immunization of two New Zealand white rabbits with a washed, heat-killed cell suspension of each of the standard organisms (17). Nine

1-ml injections (containing 10^9 organisms) were administered over a 3-week period. Both animals were bled 16 days after the last injection, and sera were stored at −20°C without further additives.

Extraction of rabbit anti-TA antibodies. Rabbit anti-TA antibody was purified by immunoabsorption at 4°C. Two portions of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) were each mixed with 1 mg of purified alpha- or beta-TA. (Residues were kindly provided by G. W. Ross. Extensive analysis of these residues had shown them to be >99% pure beta- or alpha-TA.) The TAs previously had been dissolved in phosphate-buffered saline (PBS; pH 7.2, 0.1 M) and were coupled to Affi-Gel 10 by constant end-over-end rotation for 18 h. After extensive washing of the gels (now coupled with either alpha- or beta-TA) with PBS to remove unbound antigen, two columns were prepared, each measuring 7 by 70 mm. Corresponding rabbit immune serum was passed through the column at a flow rate of 4 to 5 ml/h. Unbound serum proteins were removed by washing the columns with PBS. Elution of purified antibody from the columns was performed with 1 M propionic acid. The eluted protein was dialyzed against PBS, concentrated by evaporation, and again dialyzed. The final protein concentration was 4 mg/ml as measured by the Folin-Ciocalteu method. Purity of the eluted protein was determined by immunoelectrophoretic analysis of unabsorbed and absorbed material with goat anti-rabbit whole serum and goat anti-rabbit immunoglobulin (Microbiological Associates, Rockville, Md.). The eluted protein was stored at −20°C without additives.

CIE technique. Details of the technique have been described (11). Briefly, CIE was performed in agarose (1 g in 100 ml of 0.025 M barbital buffer, pH 8.6) and with 0.05 M barbital buffer (pH 8.6) in the electrodal reservoirs. The assay was run for 40 min. For analysis of the TA composition of the various strains of staphylococci, the crude overnight broth culture of one of the 26 strains was placed in cathodal wells in twofold dilutions in saline, and purified rabbit anti-TA antibody was placed in anodal wells. It is felt that the amount of extracellular TA produced is proportional to the amount in the cell wall itself (12, 13). In repeat assays, titers did not vary by more than one dilution.

Titration of the antibody response of the 26 patients with staphylococcal endocarditis was performed by placing serial twofold dilutions of serum in saline in the anodal wells and with one of the purified TA residues at a concentration of 10 μg/ml in the cathodal wells. The serum titers shown represent the peak titer obtained for each patient during hospitalization. Again, repeat titers varied by no more than plus or minus one tube.

Sera of patients. Initial sera of the 26 patients with staphylococcal endocarditis were obtained a mean of 7.3 days after onset of symptoms of the infection (range, 2 to 14 days). In addition, sera were obtained in some patients at weekly intervals throughout hospitalization. All sera were stored at −20°C.

RESULTS

By multiple passages through the immunoabsorbent columns, specific rabbit anti-TA antibodies were separated from whole rabbit serum. Elution of the antibody was possible with the following substances: propionic acid (1 M), sodium chloride (1 M), and acetic acid (0.1 M). Most of the antibody used in the present studies was eluted by propionic acid. Antibody precipitated in a single, clear precipitation line when CIE was performed against the corresponding antigen. When the anti-beta antibody (eluted from the beta-TA column) was assayed against the alpha residue, no precipitation was visible; thus, with this methodology there was no measurable cross-reactivity between the two TA residues and the corresponding antibodies. In addition, absorption of the eluted antibody with the corresponding soluble antigen (at equivalence, for 7 days at 4°C) resulted in complete disappearance of the precipitin line as measured by CIE. No measurable absorption was possible with the opposite antigen. Thus, great care was taken to assure and demonstrate the monospecificities of the eluted antibodies.

Immunoelectrophoretic analysis of the eluted protein with goat anti-rabbit whole serum showed two distinct precipitation lines in the area of mobility of serum immunoglobulins. A third, very faint line close to these two lines occasionally could be identified. No other serum proteins were found by immunoelectrophoresis. The identical precipitation lines were obtained with goat anti-rabbit immunoglobulins, showing that the eluted proteins were indeed immunoglobulins. Similarly, the same precipitin lines were found when pure antigen instead of the goat antiserum was used in the trough of the immunoelectrophoretic slide, showing that the immunoglobulins were antibodies specific for the TA residue in question. These data show that the eluted material consisted of TA antibodies and that these antibodies were monospecific for the alpha or beta residue used in their production.

These purified antibodies were then used for the immunochemical analysis of the 26 strains of *S. aureus* isolated from the patients with endocarditis. Extracellular TA analyses of these 26 strains are presented in Table 1. All 26 strains contained both alpha and beta residues. Each strain was prepared identically for analysis, and only moderate variation of the titer of beta-TA was observed from strain to strain. Thus, the amount of the beta residue of ribitol TA appeared to be fairly constant in these strains. Alpha-TA also occurred in all strains

TABLE 1. *Antigen concentration (in culture medium) and antibody production (in patient on day of peak titer) in S. aureus strains isolated from patients with endocarditis*

Strain no.	Beta-TA		Alpha-TA	
	Antigen[a]	Antibody[b]	Antigen	Antibody
1	1:128	1:64	1:64	1:8
2	1:64	1:64	1:64	1:32
3	1:64	1:32	1:32	1:2
4	1:128	1:16	1:2	0
5	1:64	1:64	1:32	1:1
6	1:32	1:32	1:16	1:2
7	1:64	1:16	1:8	1:2
8	1:64	1:2	1:2	1:1
9	1:32	1:4	1:16	0
10	1:64	0	1:64	0
11	1:128	1:2	1:8	0
12	1:64	1:8	1:16	0
13	1:64	1:4	1:32	0
14	1:64	1:16	1:16	1:2
15	1:64	1:32	1:64	1:8
16	1:64	1:16	1:4	1:1
17	1:128	1:16	1:8	1:1
18	1:64	1:4	1:4	0
19	1:128	1:8	1:8	1:1
20	1:128	1:32	1:32	1:4
21	1:64	1:8	1:16	1:2
22	1:64	1:4	1:2	0
23	1:64	1:16	1:32	1:2
24	1:128	1:64	1:8	1:2
25	1:64	1:16	1:32	1:2
26	1:128	1:8	1:32	0
3528	1:2	NA[c]	1:64	NA
845	1:128	NA	1:4	NA

[a] Titer of extracellular antigen in culture medium assayed by a highly purified, specific rabbit anti-TA antibody.

[b] Titer of anti-TA antibody in sera of patients with endocarditis infected with the strain of *S. aureus* listed on the left.

[c] NA, Not available.

(Table 1), but in smaller amounts and with much more strain-to-strain variation. All strains except three (strains 2, 10, and 15) contained at least twice as much beta- as alpha-TA. Some strains contained at least 16 times more beta- than alpha-TA (strains 4, 8, 11, 16, 17, 18, 19, 22, and 24). To check the method, the two reference strains of *S. aureus* were tested: strain 3528 contained almost entirely alpha-TA, and strain 845 contained all beta-TA. Control strains from non-drug users were not tested, since the broad variability in types of TAs from the tested strains did not suggest any unique properties. A previous study has shown no difference in phage types or antibiotic sensitivity patterns in strains of *S. aureus* isolated from drug users as compared with non-drug users (16).

The antibody response to either the alpha- or beta-TA residue was then assayed in the sera of the 26 patients with staphylococcal endocarditis by precipitation with the purified antigens (Table 1). In 25 of the 26 cases, a measurable antibody response was observed. Antibodies against beta-TA were found in all but one patient (patient 10, Table 1). Antibodies against alpha-TA were found in only 17 of the 26 sera (65%) and were uniformly lower in titer than those against beta-TA. Interestingly, in the nine sera containing no alpha-TA antibodies, the isolated organisms all contained alpha-TA (Table 1). In every instance where antibody was demonstrated against the test antigens, precipitins were also demonstrable against antigens from the patient's own organism.

When the antibody response was followed serially during therapy of the infection (Fig. 1), rapid disappearance with successful antibiotic therapy was observed. The beta-TA antibody remained in excess of the alpha-TA antibody throughout the course of the disease. This pattern was found in all cases where serial determinations of both types of antibodies were performed.

DISCUSSION

By immunoabsorption, purified rabbit antibodies directed against the alpha and beta residues of ribitol TA were obtained. Purified forms of both TAs are not antigenic in rabbits (5, 8); antibodies elicited by whole organisms, however, clearly reacted with purified antigens. The slight configurational differences between alpha- and beta-TA antibodies resulted in the production of two distinct antibodies; there was

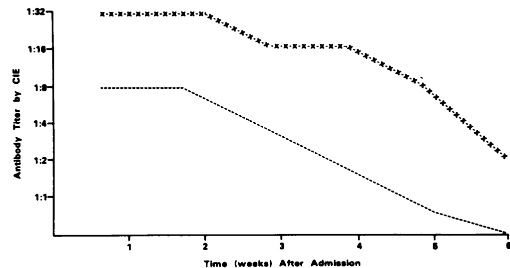

FIG. 1. *Serial determinations of both types of anti-TA antibodies, during antibiotic therapy, in a case of staphylococcal endocarditis. Antibodies against alpha-TA* (---) *were present at low titers, showed a rapid decline, and disappeared completely at 6 weeks. Anti-beta-TA antibodies* (×) *were higher in titer and persisted longer.*

no detectable cross-reaction between alpha- and beta-TA antibodies, and absorption studies with opposite antigens failed to precipitate any antibody. With antibodies thus obtained and defined, it was possible to estimate accurately the relative amounts of the alpha and beta residues produced by each of the 26 staphylococcal strains obtained from cases of endocarditis. Also, with purification of the anti-TA antibodies, it became possible to detect pure TA precipitin lines by CIE. Otherwise, when faint, such lines become lost in the multitude of precipitation lines between crude mixtures of staphylococcal antigens and unpurified rabbit antisera.

All 26 strains of *S. aureus* contained both forms of TAs, and in all instances the amount of extracellular beta-TA clearly exceeded that of the alpha residue. The two reference strains studied, both of human origin, yielded either the alpha or the beta residue, attesting to the validity of the method. Our rough estimates of the amount of antigen precipitated by each strain indicated a fairly constant content of TA from strain to strain. Our findings agree with the results of Oeding (12) in that almost all strains of *S. aureus* obtained from human infections contain beta-TA in their cell walls, and that the beta residue is also quantitatively the predominant form.

No specific distribution of the two residues of TA was found among the strains of *S. aureus* causing endocarditis in our patients. The slightly increased occurrence of alpha-TA among our strains when compared with previous reports (12) could have been due to the different technique used and to the fact that our CIE assay was more sensitive. We did not feel it necessary to study a control series of organisms acquired from other sources or patients; the patterns seen in our 26 endocarditis-associated strains seem quite representative.

In addition to taxonomic significance, this observation could be of help in developing techniques to search for circulating antigen in cases of staphylococcal septicemia. For example, after in vivo intracellular lysis of staphylococci in granulocytes, substances are released that can be detected immunoelectrophoretically and chemically resemble TAs (9); however, their immunological reactivity is unknown. The detection of circulating antigen has been used diagnostically in infections with other encapsulated organisms such as the pneumococcus (2, 4); such antigens are also polysaccharides. Thus, one might use our methods to try to look for circulating antigen in cases of staphylococcal bacteremia; however, in preliminary studies using blood and body fluid specimens from which *S. aureus* was actually culturable, we have been unable to detect any TA by either CIE or complement fixation techniques. Perhaps a radioimmunoassay would detect such antigens.

The significance of anti-TA antibodies in the diagnosis of staphylococcal infection has been described (3, 11). As with staphylococcal bacteriophages (7), teichoic acids evoke a short-lived, probably anamnestic antibody response that seems to parallel the course of the disease; it normally disappears within a few weeks after successful treatment. Control persons rarely (less than 5%) show these antibodies, and then only in undiluted serum (11). The persistence of TA antibodies may mean uncontrolled disease. After sequestrectomy in staphylococcal osteomyelitis, antibodies against staphylococcal bacteriophages fall rapidly (6).

In previous clinical reports (3, 11), no distinction was made between antibodies against alpha- and beta-TA. When we evaluated the different antibodies in the sera of our 26 patients with endocarditis, it became evident that the beta-TA antibodies were always found at higher titers and for longer periods than were alpha-TA antibodies. Antibody formation against alpha-TA was less predictable, and in some cases could not be detected despite the presence of the alpha-TA in the infecting organism. With the exception of a single case, antibodies against one or both of the TA residues were found. Thus, staphylococcal cell wall TAs are potent inducers of antibodies in humans, the beta residue more so than the alpha.

Based on these results, some practical points emerge when serological techniques are used in the diagnosis of staphylococcal infections; TA antibodies can be of significant help in rapidly diagnosing staphylococcal endocarditis. The strain of *S. aureus* used as the antigen definitely must contain beta-TA if one wishes to follow antibody titers. For future studies attempting to detect circulating staphylococcal antigens in human infections, the predominance of beta-TA among human pathogenic staphylococci must be taken into consideration.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant NIMH-7-RO1-DA-01582-01 from National Institute of Drug Abuse.

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