Counterimmunoelectrophoretic Detection of a High Incidence of Precipitin Reactions in Normal Human Sera Against Staphylococcal Teichoic Acids and Protein A

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The use of counterimmunoelectrophoresis (CIE) for detection of serum antibodies to staphylococcal teichoic acids was evaluated against teichoic acids prepared by sonic treatment or lysostaphin extraction of *Staphylococcus aureus* (Lafferty strain). Of 54 patient sera from suspected cases of staphylococcal endocarditis, osteomyelitis, or septicemia, 33 (61.1%) were positive by CIE analysis; however, 128 of 291 sera (44.0%) from normal adult donors were also positive. Selected CIE-positive sera from patient and control groups were titrated by Ouchterlony gel diffusion. In the control group of normal sera, 65% were also positive by gel diffusion, but only 15% had titers of $\geq 1:2$. Of the patient sera, 44.4% had gel diffusion titers of $\geq 1:2$. In addition to the specific teichoic acid band, a second precipitation band could be demonstrated with both patient or normal sera by CIE or gel diffusion. This second precipitin band was shown to involve interactions of test sera with staphylococcal protein A present in the teichoic acid extracts. The protein A precipitins were detected at high concentrations of the antigen extracts, whereas the anti-teichoic acid precipitins were optimally detected at lower antigen concentrations. The formation of protein A precipitin bands did not correlate with the presence of anti-teichoic acid antibodies, as most sera tested were positive for protein A regardless of anti-teichoic acid activity. This study suggests that a high incidence of normal people have levels of antibodies to teichoic acids which are detectable by the highly sensitive, but nonspecific, technique of CIE.

Since 1972 when Crowder and White (2) reported a high correlation between the presence of serum antibodies to staphylococcal teichoic acids (TA) and staphylococcal endocarditis, clinical tests to define such antibodies have received much attention. The presence of anti-TA antibodies have been reported to be of diagnostic value in assessing not only endocarditis, but also osteomyelitis and deep-seated or metastatic infections due to *Staphylococcus aureus* (4, 5, 7, 9-12). Although some serological cross-reactions have been reported between *S. aureus*, *Staphylococcus epidermidis*, streptococci, and diphtheroids, the correlation of antibodies to *S. aureus* TA with severe infections of *S. aureus* has seemed high enough to be of diagnostic value (2, 5, 10). Detection of anti-TA antibodies has generally been performed by double diffusion in agar gels (2, 5, 9-11), but more recently counterimmunoelectrophoresis (CIE) has been used for rapid screening (4, 10, 11, 12). CIE is a more

sensitive assay than gel diffusion for detection of anti-TA antibodies and, as a consequence, may be less specific.

In evaluating CIE in our clinical laboratory, we screened an initial series of seven serum samples from patients suspected of having *S. aureus* endocarditis, osteomyelitis, or septicemia. Controls for this initial series consisted of sera from 24 healthy adult volunteers. Five of the seven (72.4%) patient sera were positive by CIE analysis; however, a high incidence of positive reactions was also seen in normal sera (13 of 24 or 54.2%). Frozen samples of these sera were later assayed at Bowman Gray School of Medicine. When CIE was used with a different preparation of *S. aureus* TA, a total of 74.2% of our earlier CIE results were confirmed. In this report we have extended this early series to include 291 normal serum samples and 54 patient sera. We studied not only the incidence of positive CIE reactions against staphylococcal TA in a normal

population, but also the antigenic reactivity of TA preparations and correlations of antibody titers by CIE and gel diffusion.

MATERIALS AND METHODS

Patients and controls. During this study a total of 291 sera from normal, healthy adult volunteers were assayed for TA antibodies. A total of 54 sera were examined from patient specimens submitted to the clinical laboratories at North Carolina Memorial Hospital and Bowman Gray School of Medicine. These sera were from suspected cases of staphylococcal endocarditis, osteomyelitis, or septicemia. All patient cases were documented by pertinent bacteriological cultures. Blood cultures were performed by using both aerobic and anaerobic culture bottles on multiple samples taken at different intervals. Pertinent pathological and clinical findings were obtained from individual medical records.

Preparation of TA extracts. Preparations of TA were prepared by two different methods from overnight cultures of *S. aureus* (Lafferty strain). After the harvested bacteria were washed by centrifugation in normal saline, a 20% (vol/vol) bacterial suspension was prepared by sonic treatment by the method of Crowder and White (2). The sonically treated suspension was cleared by centrifugation at $15,000 \times g$ for 30 min and was then stored at -70°C until used. TA were also extracted by treatment with lysostaphin endopeptidase (Sigma Chemical Co., St. Louis, Mo.). Briefly, washed bacteria from overnight cultures were suspended, by volume, to 20% and treated with 5 U of lysostaphin per ml. The bacteria were centrifuged at $2,005 \times g$ for 30 min and then resuspended in the same lysostaphin-saline solution. This procedure was repeated three times. The final supernatant solution containing the extracted TA was stored at -70°C . In an effort to standardize these TA preparations, protein in the extracts was measured by the method of Lowry et al. (8). Stock solutions of each TA preparation were prepared at a concentration of 1 mg of protein per ml. When known positive sera were used, protein concentrations of 50 μg of the lysostaphin extract per ml and 200 μg of the sonically treated extract per ml gave optimal results in CIE. Specificity of the antigen extracts was checked against known positive sera and was also confirmed by analysis at Bowman Gray Medical Center.

CIE. The method of CIE was modified from that of Fedorko et al. (4). Clean glass slides (83 by 102 mm; Kodak) were coated with 10 ml of 1% agarose (Seakem; MCI, Rockland, Maine) in 0.025 M barbital buffer, pH 8.6. Paired wells 2.5 mm in diameter were cut 10 mm apart by using a Plexiglas template. Sera to be tested were placed in the anodal wells and subjected to 5 min of electrophoresis before the addition of the TA antigen suspensions to the cathodal wells. The electrophoresis buffer was 0.05 M barbital buffer, pH 8.6. CIE slides were run at 25 mA for a total of 30 min. After electrophoresis the gels were examined immediately for precipitin lines. The agarose gels were then washed for at least 24 h in saline, dried, and stained for protein with amido black. Final readings were made from the stained slides. In all CIE assays, a positive control was

included which consisted of a known positive serum or a sample of concentrated normal human immunoglobulins (Immu-G; Parke, Davis & Co., Detroit, Mich.).

Gel diffusion analyses. Gel diffusion studies were performed in 1% agarose gels made with 0.025 M barbital buffer, pH 8.6. TA extracts of *S. aureus* were used at protein concentrations of 1 mg/ml, and sera to be tested were assayed at serial twofold dilutions. TA precipitin bands were identified by the formation of lines of identity with precipitin lines formed by known positive serum. Staphylococcal protein A reactivity was identified by comparison with reactivity against purified staphylococcal protein A (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Stained gel diffusion slides were prepared by the same methods as for the CIE slides.

Removal of protein A from staphylococcal TA extracts. From gel diffusion analysis it appeared that TA extracts were contaminated with protein A. In an effort to partially purify the *S. aureus* TA, we sought to remove the staphylococcal protein A present in the extracts. Protein A was specifically absorbed from the TA extracts by utilizing the property of this protein to bind the Fc portion of immunoglobulin G (6). Polystyrene latex beads 0.81 microns in diameter were coated with purified human plasma immunoglobulin (Difco Laboratories, Detroit, Mich.). The latex beads were mixed with the immunoglobulin at a concentration of 1 mg/ml and incubated for 30 min at 37°C . The beads were then washed three times by centrifugation. For absorption of TA extracts, 1 ml of the coated latex beads was pelleted by centrifugation at $3,020 \times g$. A 1-mg amount of TA extract was added to this pellet, mixed, and equilibrated for 1 h at 4°C . After equilibration, the latex beads were removed by centrifugation. This absorption was repeated two times, and the final TA supernatant was tested by gel diffusion and CIE for TA and protein A reactivity against reference antisera.

RESULTS

Antigenic reactivity of TA preparations. Methods for detection of anti-TA antibodies have used, with only a few exceptions, relatively crude preparations of TA derived either by sonic treatment or lysostaphin extractions of staphylococcal cultures (2, 4, 7, 10). We have tried both methods for extraction of TA and have found that either approach yields preparations which vary considerably in their content of TA relative to total protein in the extract. Using known positive reference sera to standardize our preparations, we found that the optimal concentration for the formation of precipitin reactions varied greatly from preparation to preparation. For example, one lysostaphin extract of *S. aureus* gave optimal CIE precipitins at total protein concentration of 50 μg /ml, compared with a concentration of 200 μg /ml for a sonically treated extract of the same strain.

In addition to the variability in TA content, a

second precipitin band could be observed when *S. aureus* TA extracts were analyzed at high concentrations. Figure 1A shows a gel diffusion study of a lysostaphin TA extract against a known positive serum. Two distinct precipitin bands formed against the crude TA extract. Crowder and White have noted that protein A in staphylococcal extracts may give a second precipitin band in gel diffusion (2). Our study confirms this observation, as the diffuse outer precipitin band gave a reaction of identity with purified protein A (Fig. 1A). In CIE analysis of TA extracts, the protein A gave a slower-migrating diffuse band which was cathodal to the anti-TA precipitin band (Figure 1B). Extensive absorption of the TA extracts with human immunoglobulin coated on latex beads completely removed the protein A reactivity. This removal is shown by the absence of the protein A precipitin reaction when an absorbed extract was tested by gel diffusion and CIE (Figure 1A and B).

Knowledge of protein A reactivity in TA extracts is important because most sera react with protein A regardless of their anti-TA titers. Figure 2 shows a representative gel diffusion titration of two sera. Serum A was from a normal donor and gave a titer for TA of 1:4. Serum B, with a TA titer of 1:8, was from a patient with *S. aureus* endocarditis. In this example, the protein A precipitin reactions were clearly seen with both sera through the 1:8 dilutions. It has been our observation that most sera react with protein A in high dilutions (up to 1:32) regardless of their anti-TA titer.

Knowledge of protein A reactivity in TA extracts assumes even more importance in CIE determinations, because formation of either the protein A or TA precipitin bands is dependent

upon the concentration of each component. Figure 3 shows the CIE reactions of a known positive serum (Fig. 3, row A) and serum from a

FIG. 2. Gel diffusion titrations of patient and normal sera against staphylococcal TA. A lysostaphin TA extract (1 mg/ml) was placed in the center wells. Serial twofold dilutions of serum were placed in the outer wells starting with a 1:2 dilution in the top well. Serum A was from a normal adult, and serum B was from a patient with staphylococcal endocarditis.

FIG. 1. (A) Identification of protein A in TA extracts by gel diffusion. A known reference serum was applied in the center well. Purified protein A was applied to wells 1 and 4, a crude TA extract was in well 2, and a human immunoglobulin-absorbed TA extract was in well 3. (B) CIE reactivity of a TA extract after removal of protein A. A crude TA extract was applied in well 1, and an absorbed extract was applied in well 2. CIE was run against a known reference serum applied in the anodal wells.

normal adult donor (Fig. 3, row B) against three different concentrations of a lysostaphin *S. aureus* extract. At the highest total protein concentration of 250 $\mu\text{g}/\text{ml}$, both the TA and protein A precipitin bands are visible. The formation of the more cathodal protein A band was dependent upon higher total protein concentrations, as only trace reactions are observed at the lowest concentration of 50 $\mu\text{g}/\text{ml}$. Conversely, detection of the TA precipitin band is enhanced by lower antigen concentrations. At the lower concentrations of 100 and 50 $\mu\text{g}/\text{ml}$, the TA band was more distinctly formed and was farther removed from the antibody well.

Although the presence of protein A does not seem to interfere with titration of anti-TA antibodies by gel diffusion, high concentrations of protein A could conceivably mask anti-TA reactions in CIE. We have, therefore, in all of our CIE studies used carefully standardized TA preparations in concentration ranges which gave optimal TA reactivity but minimal protein A reactivity.

Incidence of positive anti-TA CIE reactions in normal and patient sera. Using a standardized lysostaphin TA extract of *S. aureus* in an initial screening of sera from 24 normal adult donors, we discovered that 54.2% of these sera were positive by CIE analysis. The specificity of the anti-TA precipitins in normal sera was confirmed by gel diffusion analysis in comparison with patient sera and a known reference serum (Fig. 4). To further test this high incidence of positive reactions in normal sera, we tested a larger series of 291 sera from normal adults as well as 54 sera from patients suspected of having staphylococcal endocarditis, osteomyelitis, or sepsis. Table 1 shows the percent-

ages of positive CIE reactions obtained against *S. aureus* TA in each category. Of the patient sera, 61% were positive; however, 44.0% of the normal sera were also positive. As far as possible, our normal serum donors were free of any active infection or disease process. These data strongly suggest that significantly high percentages of false-positive reactions can be obtained when

FIG. 4. Specificity of antibodies in patient and normal sera against staphylococcal TA. A lysostaphin extract of *S. aureus* TA was used in the center well at a total protein concentration of 1 mg/ml. Well 1 contained a known positive reference serum, wells 3 and 5 contained sera from patients suspected of having staphylococcal endocarditis, and wells 2, 4, and 6 contained samples of sera from normal adults.

FIG. 3. Concentration dependence of CIE detection of anti-TA and protein A precipitins. Antibody was applied in the anodal wells, and antigen was applied in the cathodal wells. Decreasing antigen concentrations (total protein per milliliter) were used from top to bottom: 250 $\mu\text{g}/\text{ml}$, 100 $\mu\text{g}/\text{ml}$, and 50 $\mu\text{g}/\text{ml}$. The serum in (A) was a known positive reference, and the serum in (B) was a sample from a normal adult volunteer.

CIE is used to detect anti-staphylococcal TA antibodies.

Correlations of CIE and gel diffusion anti-TA antibody titers. We selected 20 of the normal donor sera which were positive by CIE and 14 patient sera for correlation of anti-staphylococcal TA antibodies titers by CIE and gel diffusion. The 14 patient sera were selected on the basis of clinical history and the availability of bacteriological cultures for documentation of the diagnosis. The relevant clinical information for the patient specimens is summarized in Table 2, along with CIE and gel diffusion titers. The diagnoses for all patients, except patient C.B., were documented by positive bacteriolog-

ical cultures. Patient C.B. was included because of a previous history of *S. aureus* infection. Of the 14 patients, 9 had cultures confirming *S. aureus* infection. Seven of these nine patient sera were positive by CIE with titers of $\geq 1:2$. Of the seven positive CIE sera, all but one were also positive by gel diffusion. Three of the sera (patients R.B., J.D., and P.K.) with the highest CIE titers (1:8) also had significant gel diffusion titers (1:4 to 1:8). Interestingly, two sera from patients with disseminated *S. aureus* sepsis were negative for anti-TA antibodies by both CIE and gel diffusion. Four sera were included from suspected endocarditis patients in which the infections were due to *S. epidermidis* and streptococcal species. Some degree of cross-reactivity with *S. aureus* TA was observed, as all four sera were positive by CIE with titers of 1:2. Two of these sera were negative against *S. aureus* TA by gel diffusion, and two were reactive when undiluted.

Gel diffusion and CIE titers were compared in 20 normal sera which were positive by CIE screening and 9 sera from patients described in Table 2 who had culture-positive *S. aureus* in-

TABLE 1. Percentages of positive CIE reactions against *S. aureus* TA in patient and normal human sera

Serum	No. of specimens	No. positive by CIE	% positive by CIE
Patient	54	33	61.1
Normal	291	128	44.0

TABLE 2. Titers of TA antibodies in patients with staphylococcal and non-staphylococcal infections

Patient	Age	Sex ^a	Clinical diagnosis	Organism cultured	Source of organism	Underlying problems, if any	Titer of TA antibodies by:	
							CIE	Gel diffusion
R. B.	60	F	Endocarditis	<i>S. aureus</i>	Blood	Cardiovascular disease, pacemaker	1:8	1:8
J. D.	33	M	Sepsis with gangrenous abscesses	<i>S. aureus</i>	Blood	None	1:8	1:4
P. K.	26	F	Endocarditis	<i>S. aureus</i>	Blood	Drug abuse	1:8	1:8
C. H.	52	M	Sepsis with facial abscess	<i>S. aureus</i>	Blood	Chronic renal failure	1:2	1:2
M. G.	39	F	Recurrent furunculosis	<i>S. aureus</i>	Abscess	None	1:2	Negative
A. W.	30	F	Septic arthritis	<i>S. aureus</i>	Multiple lesions	Diabetes	1:2	N ^b
C. B.	60	M	Osteomyelitis	None	Joint fluid	None	1:2	Negative
G. M.	44	F	Sepsis	<i>S. aureus</i>	Blood	Adult onset diabetes	1:4	N
				<i>S. aureus</i>	Sputum			
				<i>S. aureus</i>	Tracheal aspirate			
L. J.	33	F	Pericarditis, osteomyelitis	<i>S. epidermidis</i>	Blood	Diabetes, renal failure	1:2	N
D. A.	48	M	Endocarditis	<i>S. epidermidis</i>	Blood	Diabetes, aortic valve prosthesis	1:2	Negative
B. W.	67	F	Sepsis	<i>S. aureus</i>	Blood	Chronic renal failure	Negative	Negative
				<i>S. aureus</i>	Sputum			
				<i>S. aureus</i>	Multiple lesions			
S. W.	52	F	Sepsis	<i>S. aureus</i>	Blood	Adult onset diabetes, chronic renal failure	Negative	Negative
F. H.	66	M	Endocarditis	<i>Streptococcus faecalis</i>	Abscess	Silicosis, aortic valve prosthesis	1:2	Negative
W. L.	27	M	Endocarditis	Anaerobic streptococcus	Blood	Aortic valve prosthesis	1:2	N

^a F, Female; M, male.

^b N, Undiluted.

fection. These results are summarized in Table 3. Of the CIE-positive patient sera, 44.4% had subsequent gel diffusion titers of $\geq 1:2$. These results compare with 15% of the 20 normal sera with gel diffusion titers of $\geq 1:2$. Two (22.2%) of the patient sera had gel diffusion titers of 1:8, whereas none of the normal sera gave gel diffusion titers of 1:8, and only one serum (5%) had a titer as high as 1:4. These results confirm the observation of Nagel et al. that more credibility can be given to positive CIE determinations of anti-TA antibodies if gel diffusion titers are determined (10). However, it should be noted that 65% of the positive, normal sera in our study also gave some reaction in gel diffusion.

DISCUSSION

There are three important points to be taken from this study. First, there is a high percentage of normal individuals who have some titer of antibody directed against staphylococcal TA. Second, the use of CIE, which appears to be a highly sensitive assay, can raise the detection rate of such antibody. Finally, the use of crude extracts of staphylococcal TA can further interfere with antibody detection. The precipitation of TA-antibody complexes is concentration dependent, and at high concentrations a second precipitin involving protein A may form.

Using CIE, we have detected a high incidence (44.0%) of positive reactions in our control group of 291 sera from normal adult donors. However, only 65.0% of selected sera from this group could be confirmed to be positive by gel diffusion analysis. It seems clear that CIE is a more sensitive assay than is gel diffusion and is probably also more nonspecific. Because of this nonspecificity, gel diffusion data may represent a more accurate determination of the incidence of anti-staphylococcal antibodies in the normal population. If a 65.0% confirmation is assumed, a corrected incidence of anti-TA antibodies in our pool of normal sera would be 28.6%. This incidence is much higher than that found in earlier studies evaluating the diagnostic value of anti-TA antibodies. Nagel et al. have reported an

incidence of 2.0% positive reactions within a control group of 50 healthy non-drug users (10). Although other available studies have also reported lower incidences, direct comparison is difficult because these control groups included other patients or drug users with non-staphylococcal infections (2, 7, 9, 11, 12). However, the use of different preparations of TA extracts could account for the different incidences. We have observed much variation in actual antigen concentration between different TA extracts. Because our data show that the anti-TA titers tend to be low in normal individuals, subtle antigenic differences between TA extracts may account for the variance in detection of positive reactions. It is not surprising that low titers of anti-TA antibodies can be detected in normal individuals, because Faux et al. have reported positive precipitin reactions due to exposure to environmental organic dusts containing *S. aureus* TA (3). Moreover, low-level titers could also be due to prior exposure to a variety of organisms which have been shown to cross-react serologically with *S. aureus* TA (1, 2, 5, 10).

The suggestion that differences in the detection of anti-TA antibodies may be due to the use of different preparations of TA points out a prevailing problem in all existing studies, namely, the use of crude extracts as test antigens. Unfortunately, the difficulty in obtaining staphylococcal TA prohibits their routine use in clinical laboratories. Our study stresses the need for careful standardization of crude TA extracts, as detection of anti-TA antibodies is influenced not only by the sensitivity of the assay method but also by the antigen concentration. When CIE is used, TA precipitin reactions are enhanced by low antigen concentration. This observation of concentration dependence for the specific TA reactions confirms earlier reports (3, 10), but in addition we have shown that contaminating protein A precipitin reactions may form at high extract concentrations. Substantial amounts of protein A may hinder detection of the specific anti-TA precipitins; however, simple absorptions with human immunoglobulin can

TABLE 3. Correlations of CIE and gel diffusion titers between normal sera positive for anti-TA antibodies and sera from patients with culture-documented *S. aureus* infections

Titer	Normal sera (n = 20)				Patient sera (n = 9)			
	Positive by CIE		Positive by gel diffusion		Positive by CIE		Positive by gel diffusion	
	No.	%	No.	%	No.	%	No.	%
Negative	0	0	7	35.0	2	22.2	3	33.3
Undiluted	12	60.0	10	50.0	0	0	2	22.2
1:2	6	30.0	2	10.0	3	33.3	1	11.1
1:4	1	5.0	1	5.0	1	11.1	1	11.1
1:8	1	5.0	0	0	3	33.3	2	22.2

serve as a means of partially purifying TA extracts.

The question remains as to whether determinations of staphylococcal anti-TA antibodies are of clinical value. We believe that our study has shown convincingly that CIE by itself is too sensitive and nonspecific to be useful. By using both CIE and gel diffusion, more specificity can be obtained in the determination of anti-staphylococcal antibodies. However, we believe caution must be also used in evaluating gel diffusion data, as a high percentage of our normal sera were also positive by gel diffusion. The use of titers determined by gel diffusion appears to have the most relevance to diagnosing active staphylococcal infection. Only 5% of our normal sera had anti-TA titers higher than 1:2. Therefore, titers of 1:4 to 1:8 may well indicate active staphylococcal infections. However, as Nagel et al. have suggested, the best evidence of active infection may be demonstration of a rise in antibody titer by serial assays (10).

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