Counterimmunoelectrophoresis of Staphylococcal Antibody

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Modifications of the staphylococcal counterimmunoelectrophoresis technique were evaluated to determine how variations in the procedure affected results. Neither a buffer pH range of 7.8 to 9.0 nor buffer molarity of 0.015 or 0.025 when tested in combinations caused appreciable differences. However, use of different agar preparations or delay in addition of antigen to the test slide altered the location of the precipitin band. Agarose was found to be more sensitive in determining the serum precipitin titer and provided a better photographic record than either Ionagar or Noble agar.

Counterimmunoelectrophoresis (CIE) is being utilized with increasing frequency for the diagnosis of infectious diseases (1, 3, 4). One of the most recent clinical applications has been for the detection of precipitating antibodies against staphylococcal teichoic acids in endocarditis and other infections (5, 6). However, variations in technique among different investigators raise questions about comparability of results. This is especially critical when the titer of the antibody may be significant in classifying the severity of the infection and guiding duration of therapy (5).

For example, Nagel et al., in a report on the serological diagnosis of staphylococcal endocarditis, used a CIE procedure employing 1.2% Special Noble agar in pH 8.6, 0.025 M barbital buffer with a 4- and 10-mm antigen-antibody interwell distance and a 40-min electrophoresis period (5). Parker and Fossieck, also studying the diagnosis of staphylococcal infection, utilized a CIE technique that employed 1% agarose in pH 8.6, 0.015 M barbital buffer with a 10-mm interwell distance and a 30-min electrophoresis period (6).

The purpose of this investigation was to examine modifications of the staphylococcal teichoic acid antigen CIE procedure using combinations of different agar preparations with buffers of varying pH and molarity to clarify the effects of manipulating these parameters. The results of these studies should aid in the interpretation of data presented by investigators using different systems. Additionally, laboratories wishing to use staphylococcal CIE should find the results helpful in determining an optimum procedure.

MATERIALS AND METHODS

Supporting media and buffers. Various media

and buffers combinations were tested. Three agar preparations commonly used as immunoelectrophoresis media, agarose (Sigma Chemical Co., St. Louis, Mo.), Ionagar No. 2 (Colab Laboratories Inc., Chicago, Ill.), and Special Noble agar (Difco Laboratories, Detroit, Mich.), were utilized. Gels of various concentrations were prepared in 0.015 M and 0.025 M barbital buffer at a pH of 7.8, 8.2, 8.6, or 9.0. A 0.05 M barbital buffer of the same pH as the test medium was used in the electrophoresis chamber reservoirs.

Antibody and antigen. Human serum yielding a distinct precipitin band at a 1:2 dilution in normal saline was used as the antibody source. The antigen consisted of a teichoic acid preparation from a lyso-staphin-treated culture of *Staphylococcus aureus* (Lafferty strain), kindly supplied by Arthur White. A 1:100 dilution of this preparation in normal saline consistently provided a well-delineated precipitin band with the antibody source utilized. Additional antigen dilutions of 1:200 and 1:400 were made in pH 7.0 phosphate-buffered saline. For CIE titration of the antibody, twofold serial dilutions of serum from patients with proven staphylococcal infection were made in pH 7.0 phosphate-buffered saline.

CIE. CIE was performed in a Brinkmann Sartorius Mikrophor chamber (Boskamp Geratebau KG, Goettingen, Germany) at room temperature. Current was supplied by a Brinkmann Sartorius pherostat, which could deliver either constant voltage or constant current.

Microscope slides, 25 by 75 mm, were coated with 3.0 ml of the test medium. Antibody wells, 3 mm in diameter, were positioned on the anode side of the test slide, and cathodal antigen wells of the same size were cut at various distances from the antibody wells. After addition of the antibody and antigen solutions, the slides were run in parallel at a constant 75 V with 5 mA of current per slide for 35 min. The electrical circuit was completed with Telfa (Kendall, Chicago, Ill.) wicks cut to 6.5 by 2.5 cm. The distance of the precipitin band from the antibody well was measured with a millimeter rule placed directly on the slide in a Kallestad viewer

(Kallestad Laboratories Inc., Minneapolis, Minn.) and read with a 4× magnifying glass. Both continuous, unbroken precipitin bands and discontinuous bands radiating from each side of the antibody well were considered to be positive tests. All procedures routinely used a serum of known potency as a control on each slide.

Studies. The first series of experiments was performed to determine the effect on the procedure of various antibody-antigen interwell distances in gels prepared in buffers of different pH and molarity. Gels of 1% agarose, 1% Ionagar, and 1.2% Noble agar were prepared in buffers of each molarity at the four pH levels. CIE was then performed in each gel for the eight pH-molarity combinations at interwell distances of 2, 5, 10, and 15 mm. The serum dilution was 1:2, and the antigen dilution was 1:100. All tests were performed at least four times.

To evaluate the effect of gel concentration on the procedure, a series of tests utilizing 0.5, 0.8, 1.0, 1.2, 1.5, and 2.0% agarose in pH 8.6, 0.015 M buffer was performed. The antibody and antigen dilutions were the same as in the first experiment. Interwell distance was 10 mm.

Delayed introduction of antigen into the system was also studied by using these antibody-antigen dilutions in 1% agarose prepared in pH 8.6, 0.015 M buffer. Antibody was added to each of the test slides initially. During the 35-min run, antigen was added to different slides at cumulative 5-min intervals up to 25 min after CIE was started.

Finally, the sensitivity of the three gel preparations in determining the precipitin titer of the antibody was evaluated. The gels utilized were 1% agarose, 1% Ionagar, and 1.2% Noble agar in pH 8.6, 0.015 M buffer. Twofold serial dilutions of serum from 10 patients with proven staphylococcal infection were tested against the three dilutions of the antigen preparation. Determinations of each patient's serum precipitin titer were performed simultaneously, with slides of each of the three gels run in parallel.

RESULTS

Effect of varying interwell distance and buffer pH and molarity. Table 1 depicts the location of precipitin bands in millimeter distance from the antibody (anode) well in each of the gel-buffer interwell distance combinations. The average of the distances for all buffer pH levels at each interwell distance and molarity are enclosed in parentheses.

When the table is read vertically, it can be seen that a pH range from 7.8 to 9.0 produced negligible differences in precipitin band position. This was true for the individual agar preparations at each interwell distance for both buffer molarities. However, as the distance between the reactant wells increased, there was a concomitant increase in the distance of the precipitin band from the antibody well. In some instances, at the interwell distance of 2 mm in

TABLE 1. Comparison of effect on staphylococcal CIE of different agar preparations and buffer combinations at various interwell distances^a

Interwell distance (mm)	Buffer pH	Mean band distance (mm) ^b					
		Agarose (1%)		Ionagar (1%)		Noble agar (1.2%)	
		0.015 M	0.025 M	0.015 M	0.025 M	0.015 M	0.025 M
2	7.8	0.3	0.3	0.8	0.7	0.6	0.8
	8.2	0	0	0.6	0.5	0.7	0.6
		(0.2) ^c	(0.15)	(0.8)	(0.6)	(0.8)	(0.8)
	8.6	0.1	0	0.9	0.6	1.0	1.0
5	9.0	0.4	0.3	0.8	0.6	0.9	0.9
	7.8	1.0	0.8	1.5	1.4	1.4	1.6
	8.2	0.8	0.5	1.0	1.0	1.4	1.3
		(1.0)	(0.7)	(1.3)	(1.2)	(1.5)	(1.5)
10	8.6	1.1	0.8	1.3	1.1	1.5	1.5
	9.0	1.0	0.8	1.3	1.2	1.5	1.5
	7.8	1.9	1.8	2.4	2.5	2.6	2.9
	8.2	1.5	1.4	2.1	2.1	2.5	2.5
15		(1.7)	(1.5)	(2.4)	(2.3)	(2.8)	(2.8)
	8.6	1.8	1.4	2.4	2.1	2.9	3.0
	9.0	1.5	1.4	2.5	2.4	3.2	2.8
	7.8	2.4	2.7	3.5	3.5	3.6	4.0
	8.2	2.0	1.9	2.7	2.9	3.4	3.5
		(2.2)	(2.2)	(3.3)	(3.1)	(3.8)	(3.9)
	8.6	2.2	2.0	3.3	2.9	3.8	4.3
	9.0	2.3	2.0	3.5	3.2	4.4	3.8

^a When read vertically, precipitin band position is compared in each gel at various interwell distances. Horizontally, band position at a single interwell distance is compared between gels.

^b Mean distance of the precipitin band from the antibody well at each pH.

^c Numbers in parentheses are mean band positions in millimeters at each interwell distance.

agarose, the precipitin band appeared at the edge of the antibody well. At the largest interwell distance of 15 mm, the precipitin bands were often wide and indistinct.

When Table 1 is read horizontally, the position of the precipitin bands in each of the three gels is compared at each interwell distance. It is evident that the agar preparation itself influences reactant migration and band position, with the greatest absolute differences present at the largest interwell distance. However, at the greater distances, the relative differences in band position are proportionally less.

Several additional systems not depicted in Table 1 were tested: a continuous one using 0.25 M barbital buffer at pH 8.2 in both the chamber reservoir and medium and a discontinuous system using pH 8.6, 0.075 M barbital buffer in the reservoir chamber and pH 8.6, 0.015 or 0.025 M

barbital buffer in the medium. Results with these systems were similar to those shown in Table 1.

Varying gel concentrations. Table 2 illustrates the results of varying the concentration of the gel on the position of the precipitin band. Precipitin band position was the same at all concentrations except the lowest. The lowest concentration (0.5%) was unsuitable, however, due to lack of sufficient solidity.

Delay in antigen introduction. In addition to varying the above parameters, delayed introduction of antigen into the CIE system was also studied. Figure 1 shows the average results of such experiments. There was a linear relationship between the interwell position of the precipitin band and the addition time of the antigen. The greater the delay prior to addition of the antigen, the greater was the distance of the precipitin band from the antibody well.

In the 35 min allowed for the reaction, a delay of 25 min prior to addition of the antigen frequently resulted in the precipitin bands forming after the run was terminated and while the slide was being observed in the viewer. It can be estimated from Fig. 1 that the teichoic acid antigens from *S. aureus* migrate at a rate of about 0.2 to 0.25 mm/min in this system. Antibody apparently migrates only

about one-half as rapidly. Except for the position of the precipitin band, stopping the test to apply the antigen did not appear to affect results.

Sensitivity in determining precipitin titer. Finally, the sensitivity of the three agar preparations in determining the titer of the antibody was evaluated. Results are summarized in Table 3, and a representative test series is illustrated in Fig. 2.

Whereas titration could be successfully performed in each of the gels, the titers obtained in agarose were higher by one dilution or more, except in one instance when the agarose and ionagar titers agreed (patient 4). The titers of the serum precipitins were the same for each of the three dilutions of antigen preparation. At the initial dilution of 1:100, discontinuous bands radiating from each side of the antibody well occurred with some of the sera in agarose only. The tests using the two other antigen preparation dilutions resulted in continuous bands separate from the antibody well in each of these instances. The three slides in Fig. 2 were photographed simultaneously side by side. Although the titer appears higher in agarose at the 1:16 dilution, the results were 1:16 in both Noble agar and Ionagar and 1:32 in agarose. The photographic reproduction is clearly superior in agarose.

DISCUSSION

Staphylococcal teichoic acid antigen CIE for detection of antibody has proven to be a useful adjunct for the diagnosis of staphylococcal infection (5, 6). With the limited information currently available, it has been suggested that the titer of the antibody may be related to the type and severity of disease (5). If this proves to be the case, either a standardized method of titer determination must be developed or a means of comparing results between different CIE procedures must be derived.

TABLE 3. Comparison of staphylococcal CIE precipitin titers in different gels

Patient	Reciprocal of serum dilution		
	Agarose	Ionagar	Noble agar
1	32	16	16
2	32	16	16
3	16	8	4
4	8	8	4
5	8	4	4
6	8	4	4
7	16	8	8
8	16	4	4
9	32	16	16
10	8	4	4

TABLE 2. Influence of gel concentration on precipitin band position at a 10-mm interwell distance

% Agarose concn	Mean band distance (mm) from antibody well
0.5	1.1
0.8	1.5
1.0	1.6
1.2	1.6
1.5	1.5
2.0	1.5

FIG. 1. Effect of delayed addition of teichoic acid antigen on position of precipitin band.

FIG. 2. Demonstration of teichoic acid antibody titers simultaneously determined by CIE in three agar preparations. Actual titer by direct visualization was 16 (reciprocal of serum dilution) in Noble agar and Ionagar and 32 in agarose.

In the CIE system used in this study, manipulation of various parameters was evaluated to determine the influence upon the end result. Alteration of pH from 7.8 to 9.0 in barbital buffer of either 0.015 or 0.025 molarity did not appreciably affect precipitin band clarity or position with the reactants utilized. In addition, varying the concentration of the gel in the buffer from 0.5 to 2.0% also produced no noticeable differences. However, gel of 0.5% concentration lacked sufficient firmness to be useful.

Two other factors, though, the type of agar preparation and delay of introduction of the antigen into the system, resulted in clearly demonstrable differences. In the first instance, utilization of a very purified agar preparation with low endosmotic activity, agarose (2), led to precipitin bands positioned nearest the antibody well. The differences were most apparent at the greater interwell distances, with the precipitin band farthest from the antibody well in Noble agar, intermediate in Ionagar, and closest in agarose. At the shortest interwell distance of 2 mm in agarose; the bands frequently formed at the edge of the antibody well. This supports the observation of others that false negative results may even occur if precipitation occurs in the antibody well at short interwell distances due either to the high electrophoretic mobility of the teichoic acids or to the occurrence of antigen excess (5). However, precipitin

bands were most distinct at short and intermediate interwell distances, with broad indistinct bands forming at the 15-mm distance.

The second factor, delayed application of the antigen, produced the most striking results. If CIE was begun with antibody already in the anode well, delay in addition of antigen into the cathode well for 5-min intervals up to 25 min allowed development of the precipitin band at any desired interwell location. The longer the delay prior to addition of the antigen, the further was the distance of the precipitin band from the antibody well. This procedure of delayed antigen addition can be advantageously used with sera producing a diffuse nonspecific background haze near the antibody well that might obscure weak precipitin bands forming close to the well.

Finally, tests of the sensitivity of the three agar preparations in detecting the titer of antibody revealed additional differences between the gels. Agarose was the most sensitive when the antibody titer of each of 10 patients with proven staphylococcal infection was determined in the three gels simultaneously. A difference of as great as two dilutions higher was detected with agarose. The possibility therefore exists of a one or greater dilution difference in antibody titer between studies utilizing agarose and Noble agar or Ionagar. This potential discordance could be of import if titers prove to be of differ-

ential diagnostic significance. Agarose also produced clearer, more distinct, and easily detectable precipitin bands when identical titers in the three gels were compared. An additional advantage was the superior photographic reproducibility of results from the agarose slides.

Although we have not evaluated the use of these three agar preparations in initial diagnosis, agarose has the potential for positive results at low titer when other gels may appear negative. Therefore, when evaluating results of investigations utilizing different CIE systems, the reader must be aware of the possible variability induced by the procedures.

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