Practical Considerations in Using Counterimmunoelectrophoresis to Identify the Principal Causative Agents of Bacterial Meningitis

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Received for publication 24 July 1979

Many clinical laboratories are currently using counterimmunoelectrophoresis (CIE) as an aid in the rapid diagnosis of bacterial meningitis. Because cross-reactions among causative agents have been reported, the present study was undertaken to explore the problems that might occur when reference and commercial antisera are used in CIE. Broth cultures of 35 bacterial strains were tested with 76 reference and commercial antisera by CIE. Some of the antisera tested failed to react with their homologous strains. Furthermore, several cross-reactions between genera, as well as within species, were noted. These findings suggest that precautions must be taken to insure that all materials used in CIE tests are of high quality. If properly performed and interpreted, CIE may be a valuable adjunct in the identification of organisms causing bacterial meningitis, but it is, nevertheless, a presumptive test and should not be used to replace the Gram stain and culture techniques.

The need to diagnose bacterial meningitis as quickly as possible has led to the use of rapid diagnostic techniques that detect bacterial products in cerebrospinal fluid (CSF). Counterimmunoelectrophoresis (CIE) has been used to detect in CSF the soluble polysaccharide antigens of Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae, group B Streptococcus, and Escherichia coli K1 (5, 11, 15, 16, 18, 27). Several investigators studied the sensitivity of CIE and emphasized the necessity of using antisera with high titers of precipitating antibodies (5, 7, 41). The positivity rates by CIE of 779 CSF specimens from patients with meningococcal, pneumococcal, or H. influenzae meningitis were 75, 79, and 84%, respectively (4–6, 8, 11, 15, 16, 19, 30, 31, 39, 40–42). The specificity of the technique was also studied, and some cross-reactions were reported (17, 33). Such cross-reactions are not surprising because some of the organisms are known to have antigenic similarities (3, 14, 29, 35, 37).

Our study was designed to explore the problems that might occur when reference and commercial antisera are used to detect antigens of organisms causing bacterial meningitis. Although the technique is being used widely, few antisera are currently prepared or evaluated specifically for use in CIE. We hoped that any cross-reactions observed in our study might serve as a caveat to laboratorians using CIE to help establish a diagnosis of bacterial meningitis. Moreover, since interest in determining serological groups or types for epidemiological purposes has increased, these findings may have some bearing on the use of CIE for grouping or typing isolates of these organisms.

MATERIALS AND METHODS

Bacterial strains and antisera. Representative stock strains of each of the following bacteria, obtained from culture collections at the Center for Disease Control, Atlanta, Ga., and retested for purity and correct identity, were used to evaluate the specificity of 76 antisera for CIE testing: group B Streptococcus types Ia (strain 090), Ib (H36B), Ic (A909), II (SM821), and III (D136C); S. pneumoniae types 1 (SP1), 3 (SP43), 4 (SP4), 6 (SP73), 7 (SP70), 8 (SP46), 12 (SP12), 14 (SP14), 18 (SP18), 19 (SP19), 22 (SP76), and 23 (SP23); H. influenzae types a (SM4), b (GB329, E796), c (SM72), d (SM6), e (SM7), and f (SM8); N. meningitidis groups A (1894), B (2091, E746), C (1166), and Y (KC661); Staphylococcus aureus (1499), Listeria monocytogenes (E340); and E. coli strains O1:K1:NM (1721-76), O7:K1:NM (764-64), O13:K9:H4 (4113-73), and O7:K92:H54 (6181-66).

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purchased from Burroughs-Wellcome Corp.; Difco Laboratories; Hyland Laboratories, Inc.; and the Statens Seruminstitut. With the exception of two equine group B meningococcal antisera, all antisera were produced in rabbits. Specificities and numbers of sources (in parentheses) were as follows: group B Streptococcus (three); S. pneumoniae types 1 (three), 2 (two), 3 (two), 6A (four), 7 (two), 12 (one), 14 (two), 18 (two), 19 (two), 22 (one), and 23 (one); S. pneumoniae polyvalent (one); H. influenzae types a (four), b (four), c (four), d (four), e (four), and f (four); H. influenzae polyvalent (one); N. meningitidis groups A (three), B (four), C (three), D (two), X (two), Y (three), and Z (two); N. meningitidis polyvalent for groups X, Y, and Z (one).

Antigen preparation. Frozen blood suspensions of the organisms were allowed to thaw and then were transferred to a suitable growth medium: 5 ml of Todd-Hewitt broth supplemented with 0.5 ml of defibrinated rabbit blood for streptococci and pneumococci; 5 ml of heart infusion broth supplemented with 0.5 ml of defibrinated rabbit blood for S. aureus, L. monocytogenes, and E. coli; 5 ml of heart infusion broth supplemented with 2 drops of peptic digest of blood for H. influenzae; and a chocolate agar plate (heart infusion agar base with supplement B) for meningococci. After overnight incubation at 35°C (in candle extinction jars for pneumococci, meningococci, and H. influenzae, and in a loopful, 3-mm diameter) of each broth culture was transferred to a second 5 ml of broth (same kind of broth as used for the first culture; no supplements except 2 drops of peptic digest of blood for H. influenzae). The second broth was incubated as before for 20 h. For the meningococci, a heavy inoculum from growth on the 24-h plate was transferred to 2 ml of heart infusion broth, which then was incubated for 20 h at 35°C in a candle extinction jar. The 20-h broth suspensions contained 10^9 to 10^10 colony-forming units per ml (except for one group B meningococcal broth that contained 2 × 10^9 colony-forming units per ml and one type 14 pneumococcal broth that contained 4 × 10^9 colony-forming units per ml). These broth suspensions were used to fill antigen wells in the CIE experiments described below.

CIE. Precast glass Kodak slides (3/4 by 4 in. [ca. 8.26 by 10.16 cm]) were covered with 12.5 ml of 1% agarose (SeaKem ME, lot no. 62976, Marine Colloids, Inc., Springfield, N.J.) in Veronal buffer (pH 8.6; ionic strength, 0.05). Parallel rows of circular wells, 3 mm in diameter, were cut in the agarose gel 3 mm apart, edge to edge. Each anodal well was filled with approximately 10 μl of antigen, and each cathodal well was filled with approximately 10 μl of uncentrifuged, 20-h broth culture or, as a control, un inoculated broth. Slides were placed in a chamber (Helena Laboratories, Beaumont, Tex.) containing 150 ml of Veronal buffer (pH 8.6; ionic strength, 0.05) in each reservoir; electrophoresis was allowed to proceed at room temperature for 30 min at a constant current of 20 mA per Kodak slide. Unstained slides were examined by incident light for precipitation lines immediately after electrophoresis and then at least twice again during 24-h storage in a humid chamber at 4°C.

In a typical CIE test, the same broth culture or un inoculated broth sample was used to fill all of the antigen wells, and 76 different antisera were used to fill the antisera wells.

Cross-reactions or questionable weak reactions observed in the original tests were verified by repeating the tests. When precipitation lines occurred between an antiserum and several heterologous strains, the strains were grown in a different broth medium and retested by CIE as described above. Broths used as alternates included: Trypsincase soy broth (BBL Microbiology Systems) for streptococci, pneumococci, E. coli, S. aureus, and L. monocytogenes; Schaedler broth for H. influenzae; and Mueller-Hinton broth for meningococci. Alternate broths were supplemented with peptic digest of blood or with defibrinated rabbit blood as described above for the broths used in the original tests.

RESULTS

Three group B streptococcal antisera correctly reacted with the five group B streptococcal strains representing types Ia, Ib, Ic, II, and III. All reactions occurred within 8 h except one; this reaction, between an antiserum and the type III strain, was not observed until after overnight incubation at 4°C. Two of the group B streptococcal antisera reacted with several heterologous strains and occasionally with an uninoculated broth. When these strains were grown in alternate broths, no lines were observed between antiserum and broth cultures or uninoculated broths.

H. influenzae type-specific antisera (types a through f) from four sources produced precipitation lines with their homologous type strains. All reactions were visible within 8 h with one exception, in which case type c antiserum did not produce a visible line with its homologous strain until after overnight incubation at 4°C. One lot each of type c and d antisera also reacted with strains of types a and f, respectively. Cross-reactions between H. influenzae type-specific antisera and bacteria of different species were observed; these are described below.

One H. influenzae polyvalent antisera was tested by CIE. It reacted with antigen in broth cultures of types b, c, and f (type f only after overnight incubation at 4°C) but failed to react with antigen in broth cultures of types a, d, and e.

Immediately after electrophoresis, precipitation lines were observed between meningococcal antisera for groups A, C, and Y (three lots each) and their homologous strains. Four lots of group B meningococcal antisera were tested; three of these produced visible lines with both group B strains within 8 h, whereas the fourth failed to react with the group B strains, even after overnight incubation at 4°C. A surprising number of intraspecies and intergeneric cross-reactions (de-
scribed below) were observed.

The meningococcal polyvalent antiserum for groups A, B, C, and D failed to react with N. meningitidis group A but did react with strains of groups B and C within 8 h. The polyvalent antiserum for groups X, Y, and Z reacted with the group Y strain included in the study.

One polyvalent and 26 type-specific pneumococcal antiseras were tested with 35 bacterial strains, including 12 pneumococcal strains. Each type-specific antiserum reacted with its homologous strain (including the broth that contained <10⁷ type 14 colony-forming units per ml), although one type 12 antiserum did not react with the type 12 strain until after overnight incubation at 4°C. The polyvalent antiserum reacted with 11 of 12 strains immediately and with the type 3 strain within 3 h.

Both intraspecies and intergeneric cross-reactions were observed with pneumococcal antiseras and will be described below. One pneumococcal type 7 antiserum reacted with unincubated Todd-Hewitt broth and heart infusion broth and with every strain grown in either of these two broths. When strains were grown in alternate broths, no precipitation lines were observed, suggesting that the antiserum had reacted with components of Todd-Hewitt broth and heart infusion broth in the original tests.

It was of interest that pneumococcal type 7 and 14 antiseras reacted with their homologous strains, because these capsular antigens are reportedly neutral (2). With Center for Disease Control type 7 and 14 antiseras, precipitation lines were observed very close to the antigen wells, which implies that antigen had diffused passively into the medium; in addition, a centrally located line occurred between the type 14 antiserum and its homologous strain.

As indicated above, numerous intraspecies and intergeneric cross-reactions were observed in the present study. These have been summarized in Tables 1 and 2. For example, antiseras of H. influenzae types c and d (one lot each) reacted with strains of H. influenzae types a and f, respectively (Table 1). The reaction was rapid and the precipitation lines were intense. The most notable of the cross-reactions among the meningococcal antiseras and strains were those between an N. meningitidis group A antiserum and group B meningococcal strains (two strains tested), between an N. meningitidis group B antiserum and an N. meningitidis group C strain, and between an N. meningitidis group D antiserum and group B meningococcal strains (two strains tested). In fact, if the intensities of these reactions were compared with those of the homologous reactions, one group B meningococcal strain might have been identified as N. men-

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Heterologous strain giving positive CIE reaction</th>
</tr>
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<tbody>
<tr>
<td>H. influenzae c</td>
<td>H. influenzae a</td>
</tr>
<tr>
<td>H. influenzae d</td>
<td>H. influenzae f</td>
</tr>
<tr>
<td>N. meningitidis A</td>
<td>N. meningitidis B, C</td>
</tr>
<tr>
<td>N. meningitidis B</td>
<td>N. meningitidis C</td>
</tr>
<tr>
<td>N. meningitidis D</td>
<td>N. meningitidis B</td>
</tr>
<tr>
<td>N. meningitidis Z</td>
<td>N. meningitidis B, C</td>
</tr>
<tr>
<td>S. pneumoniae 2</td>
<td>S. pneumoniae 7, 12, 14, 22</td>
</tr>
<tr>
<td>S. pneumoniae 3</td>
<td>S. pneumoniae 7, 12</td>
</tr>
</tbody>
</table>

a Lower-case letters and numbers denote serological type. Upper-case letters denote serological group.

b Numerous intraspecies cross-reactions were observed among pneumococcal antiseras and strains. Only those that would have resulted in misidentification have been included.

ingitidis group A by one set of antiseras, and both group B meningococcal strains would just as likely have been identified as N. meningitidis group D by another set of antiseras.

Many intraspecies cross-reactions were observed among pneumococcal antiseras and strains. By paying close attention to the intensity and rapidity with which precipitation lines formed, it would have been possible to identify most pneumococcal strains correctly by their reactions with type-specific antisera; several strains, however, would have been identified incorrectly. With one set of antiseras, S. pneumoniae types 7 and 12 probably would have been identified as type 3, and with another set of antiseras types 7, 12, 14, 22 and 26 probably would have been identified as type 2. The latter reactions are included in Table 1.

Intergeneric cross-reactions observed in the present study are shown in Table 2. Among the most notable of these were reactions between an N. meningitidis group B and E. coli strains O7: K1:NM, O13:K92:H4, and O73:K92:H34, between an H. influenzae type b antiserum and an S. aureus strain, and between an S. pneumoniae type 19 antiserum and H. influenzae type a.

**DISCUSSION**

Bacterial broth cultures were used as antigens in the present study because the release of soluble antigen in broth seemed more likely to mimic its release and subsequent detection in CSF than would the extraction and concomitant degradation of antigens by chemical or by physical means. The concentrations of organisms used in the present study (10⁷ to 10⁹ colony-forming units per ml) were in the upper range of bacterial concentrations likely to be found in
TABLE 2. Intergeneric cross-reactions occurring in CIE tests

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Heterologous strain giving positive CIE reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. influenzae b</td>
<td>S. aureus</td>
</tr>
<tr>
<td>H. influenzae e</td>
<td>N. meningitidis B</td>
</tr>
<tr>
<td>H. influenzae f</td>
<td>N. meningitidis C</td>
</tr>
</tbody>
</table>
| N. meningitidis B | E. coli 013:K92:H4
| N. meningitidis C | E. coli 013:K92:H4 |
| S. pneumoniae 3 | N. meningitidis B b |
| S. pneumoniae 19 | H. influenzae a |
| S. pneumoniae polyvalent | N. meningitidis B b |

* Lower-case letters and numbers denote serological type. Upper-case letters denote serological group.

Reactions visible after at least 8 h of incubation at 4°C.

CSF, according to published reports (9, 10). However, the concentration of soluble antigen in CSF of a patient might not be reflected accurately by the bacterial count, particularly if the clinical course has been lengthy. High concentrations of organisms were chosen in the present study to reveal the greatest number of cross-reactions that might occur.

The necessity of testing the sensitivity of antisera was emphasized by the failure of some monospecific antisera to react with homologous strains within 8 h after electrophoresis. Because either antigen excess or antibody excess is possible in tests with CSF or broth cultures, the testing of twofold dilutions of either or both reactants should be considered. One group B meningococcal antiserum did not produce precipitation lines with either of two group B meningococcal strains tested, even after overnight incubation at 4°C. Problems with group B meningococcal antiserum were observed by others. In 1972, Tobin and Jones (41) reported that commercial group B meningococcal antiserum detected no antigen by CIE in 22 clinical CSF specimens, 10 of which yielded N. meningitidis group B in culture. More recently, Ghanassia et al. (12) reported that no specific precipitation lines were observed with 28 CSF specimens that were culture positive for N. meningitidis group B.

The H. influenzae polyvalent antiserum did not appear to be adequate for detecting all of the type-specific H. influenzae antigens. If an H. influenzae polyvalent antiserum is to be relied on, it must have a high titer of precipitating antibodies to H. influenzae type b, because type b is the principal cause of bacterial meningitis in children between the ages of 1 month and 3 years in the United States (38).

It is interesting that a meningococcal polyvalent antiserum for groups A, B, C, and D reacted with antigen in broth cultures of group B strains, whereas the monospecific antiserum for group B meningococcal antigen from this company failed to do so. It appears that the titer of precipitating antibodies to group B polysaccharide antigen was higher in the polyvalent antiserum than in the monospecific antiserum.

Several intraspecies and intergeneric cross-reactions were noted in the present study. The cross-reactions may be explained by one or more of the following: (i) immunochemically similar type or group capsular antigens that are bound loosely to the bacterial cell surface; (ii) similar noncapsular antigens that are released into the media during growth or by lysis of the organisms; (iii) broth constituents in the suspending medium that are chemically similar to bacterial antigens; and (iv) broth constituents which are inadvertently present in vaccine preparations and which serve as antigens during antiserum production.

The first possibility may be supported in some instances by proposed structural similarities (Table 3). The observed cross-reactions of N. meningitidis groups B and C with E. coli strains K1 and K92, respectively, may have been due to common N-acetylneuraminic acid determinants in the former and common sialic acid determinants in the latter. Similarly, the cross-reactions between an H. influenzae type b antiserum and an S. aureus strain most likely occurred because

TABLE 3. Organisms with cross-reactive capsular polysaccharide antigens as reported in the literature

<table>
<thead>
<tr>
<th>Organism</th>
<th>Possible cross-reacting structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. meningitidis B/E. coli O7: K1(L):NM</td>
<td>N-Acetylneuraminic acid</td>
<td>22, 36</td>
</tr>
<tr>
<td>N. meningitidis C/E. coli K92</td>
<td>Sialic (neuraminic) acid</td>
<td>13</td>
</tr>
<tr>
<td>H. influenzae b/S. aureus subsp. copenhagen</td>
<td>Polyribitol phosphate</td>
<td>3</td>
</tr>
<tr>
<td>H. influenzae b/E. coli O7: Kf147:H5</td>
<td>Acidic polysaccharide</td>
<td>36, 37</td>
</tr>
<tr>
<td>E. coli O48:NM/S. pneumoniae 1</td>
<td>Acidic polysaccharide without detectable lipids or protein</td>
<td>35, 36</td>
</tr>
<tr>
<td>E. coli O7:H4/K7/S. pneumoniae 3</td>
<td>Acidic polysaccharide</td>
<td>35, 36</td>
</tr>
</tbody>
</table>

* Now designated E. coli O75:K100:H5 (32).
of common polyribitol phosphate determinants. Also listed in Table 3, but not tested in this study, are reported cross-reactions between several other E. coli capsular types and pneumococcal types 1 and 3 and H. influenzae type b.

Some of the observed or expected cross-reactions have been described in clinical situations. Investigation of the antigenic relationship between N. meningitidis group B and E. coli O7: K1(L):NM was begun after a bacterial strain isolated from the CSF of an infant with meningitis was identified as N. meningitidis group B and then later found to be E. coli O7:K1(L):NM (14). Group B meningococcal antiserum has been used to detect E. coli K1 antigen in CSF and serum of neonates with meningitis (34). In another case, H. influenzae type b antiserum reacted with antigen in the CSF of a patient with S. aureus endocarditis (33).

Intraspecies cross-reactions may also be explained by antigenic similarities. For example, group A meningococcal polysaccharide is a polymer of N-acetyl, O-acetylmannosamine phosphate (25), group B polysaccharide consists mainly of N-acetylmuramic acid, and group C polysaccharide consists mainly of N-acetyl, O-acetylmuramic acid (24). N-Acetylmannosamine 6-phosphate is an intermediate in the biosynthesis of muramic acid (23). Therefore, it seems possible that the polysaccharide antigens of N. meningitidis groups A, B, and C may have common determinants. Because of the spatial configurations of the antigenic determinants on whole cells, these sites might be exposed in a precipitation test but not in a test such as slide agglutination. Furthermore, many strains of meningococcal groups A, B, C, and Y reportedly have antigenically similar surface serotype protein antigens (21).

Similarly, the type antigens of H. influenzae types a, b, c, and f have a common structural feature consisting of (1→1)-linked disaccharide subunits bridged by phosphodiester groups (43); the type d antigen is a polysaccharide that does not contain phosphorus or sulfur (1). In addition, all of the types possess a common protein antigen (28); this so-called M substance is a labile surface antigen (20).

Many of the cross-reactions among the pneumococcal antisera and strains may have been due to similar type-specific polysaccharide antigens or, in support of the second possibility mentioned above, they may have been due to common noncapsular antigens. A cell wall carbohydrate antigen, known as "C substance," occurs within all types of the species (26).

The possibility that an antiserum might react with broth constituents present in the suspending medium may account for the cross-reactions observed with a pneumococcal type 7 antiserum and, perhaps, with two of the group B streptococcal antisera. Such reactions emphasize the importance of including medium controls in CIE tests when the antigens are suspended in media.

Precautions such as the following must be taken when CIE is being used to help in the identification of organisms causing bacterial meningitis: (i) antisera must always be tested for sensitivity and specificity; (ii) antigen controls should be included in CIE tests regularly; (iii) uninoculated broth (medium) controls should be used to test the specificity of antisera if CIE is being used as a grouping or typing technique for organisms grown in broth or harvested in medium; and (iv) because of potential serological cross-reactivity, culture and microscopy data should be used in interpreting positive CIE results.

In conclusion, if properly performed and interpreted, CIE may be a valuable adjunct in the identification of organisms causing bacterial meningitis, but it should not be used to replace the Gram stain and culture techniques.

ACKNOWLEDGMENT

This study was supported by Public Health Service Special Purpose Traineeship 5A04 02119-03.

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