Technical Factors Affecting an Immunoelectrophoretic Reference System for Analysis of Mycobacterial Antigens

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The length of immunoelectrophoretic separation patterns obtained with a reference mycobacterial antigen was reduced significantly when a continuous buffer system was used in place of the recommended discontinuous system. Varying the supporting medium by using 1% concentrations of different agar preparations in the discontinuous system also affected the separation length and resolution of the reference pattern. Measurement of the electrical resistivity of the agar medium provided an explanation of these differences on the basis of current density or field intensity applied per slide. Variation in the reference pattern obtained in differently designed electrophoresis chambers also was attributed to differences in field intensity. To avoid these obvious alterations in the separation pattern of the reference antigen, it was suggested that separation procedures described originally for the reference system be used without modification.

It has been suggested (4) that an immunoelectrophoretic reference system could facilitate the identification and characterization of mycobacterial antigens. With this system, antigenic components isolated in different laboratories, by different fractionation procedures, and from different starting materials could be related to each other and to the components of a reference antigen. The ability to compare antigens in this manner also might provide the means to develop an antigenic taxonomy for the mycobacteria and to identify species-specific antigens. However, it must be emphasized that the full potential of a reference system can be realized only if comparability in immunoelectrophoretic methodology among laboratories is guaranteed. The availability of various agar preparations, buffers, and types of electrophoretic equipment raises the possibility of numerous methodological variations which might alter the separation pattern of the reference antigen. The purpose of this report is to describe the influence of representative variations in separation methodology on an immunoelectrophoretic reference system for mycobacterial antigens and to identify factors critical for maintaining comparability of separation methodology.

MATERIALS AND METHODS

Antigen and antiserum. The reference antigen (lot no. 001) and reference antiserum (lot no. 001) used in all experiments were provided in a mycobacterial reference kit which was obtained through the United States-Japan Cooperative Medical Science Program from the Geographic Medicine Branch of the National Institute of Allergy and Infectious Diseases (Bethesda, Md.).

Immunoelectrophoretic analysis. As described for the reference system (4), electrophoretic separation of the reference antigen was conducted in a Buchler apparatus (Fort Lee, N.J.) on glass microscope slides (1 by 3 inch; ca. 2.5 by 7.6 cm) coated with 2 ml of buffered agar. Exact placement of the wells and troughs in the agar gel was accomplished with a Buchler agar cutter. All electrophoretic separations were conducted in pH 8.6 barbital buffer at room temperature (25°C) for 90 min, and a constant current of 1.67 mA was applied per slide. Immediately after separation, the reference antiserum was added to the troughs, and then the slides were incubated in a humid chamber at room temperature for 24 hr, at which time the immunoelectrophoretic separation patterns were photographed.

In experiments designed to determine the influence of the buffer system, the separation patterns obtained with the reference materials in the discontinuous buffer system, prepared as described (4), as well as in a continuous buffer system, prepared using
the formulation of the buffer for the electrode vessels (4), were compared. Similarly, to determine the influence of the agar, separation patterns of the reference antigen in 1% Ionagar no. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.), Noble agar (Difco), purified agar (Difco), and agarose (Mann Research Laboratories, Orangeburg, N.Y.) were compared. Merthiolate was added to the buffered agar solutions at a final concentration of 1:10,000. Also, when the influence of the design of the apparatus was studied, patterns obtained with the antigen separated on slides on a Buchler slide carrier were compared with those obtained on an LKB slide carrier (LKB Instruments, Inc., Rockville, Md.) in the Buchler apparatus. The design of the slide carrier was examined, rather than the entire electrophoresis apparatus, because most units have essentially similar construction and usually vary only in the configuration of the slides within the separation chamber. The Buchler slide carrier has a parallel placement of slides, and the LKB carrier utilizes a series-parallel combination of slides. In this comparison, electrophoretic separation of the antigens was conducted in 1% Ionagar using a discontinuous buffer system at a constant current of 10 mA per slide carrier for 90 min.

In all of these comparisons, six slides were prepared for each variation; the reference antigen was placed on each slide, and all six slides were subjected to electrophoresis simultaneously within a single chamber. To quantitatively compare immunoelectrophoretic patterns, the distance between the leading end of the most anodic band and the trailing end of the most cathodic band was measured on coded photographs. The significance of differences between mean separation lengths was analyzed statistically using a small-sample t test (3).

Resistivity determinations. A half-bridge apparatus (6) was constructed, as shown diagrammatically in Fig. 1, to determine the resistance of the various buffered-agar preparations. The sample chamber was a polystyrene test tube (16 by 150 mm) into which the four electrodes were cemented. The electrodes were prepared from 0.025-cm silver wire (Medwire Corp., Mt. Vernon, N.Y.), to minimize polarization, the wire was plated, using the methods described by Janz (5), to form silver-silver chloride electrodes. The current electrodes (E₁ and E₄) were positioned at the top and bottom of the tube, 11 cm apart, across its diameter, and the potential electrodes (E₂ and E₃) were located 5 cm apart approximately mid-way between the current electrodes. For actual measurements, approximately 19 ml of buffered agar was poured into the tube; it was allowed to harden and equilibrate to room temperature. A sinusoidal current then was applied over a frequency range of 0.05 to 100 Hz from a signal generator (model 202A, Hewlett-Packard Co., Palo Alto, Calif.) to the sample chamber through the current electrodes, and the voltage drop across the sample was detected by the potential electrodes. After amplification and filtering, the resistance of the sample was balanced by adjusting Rs, a variable resistor (model 1432-M, General Radio Co., Concord, Mass.). Thus, when a null output was obtained, the value of Rs served as a direct measure of the sample resistance. Repeated measurements of resistance in the same chambers indicated that the precision of measurement was within 1%. A separate chamber was made for each agar preparation; variation between chambers was corrected by calibrating the chambers with 0.9% sodium chloride before use.

The resistance of the sample was converted to resistivity by the formula \( \rho = R (A/L) \) in which \( \rho \) is the resistivity in ohm-cm, \( R \) is resistance in ohms, \( A \) is the cross-sectional area of the sample chamber in cm², and \( L \) is the distance between the potential electrodes in cm; \((A/L)\) was a constant value of 0.266 cm for each chamber. The field intensity in millivolts per centimeter, developed on a slide during electrophoretic separation, was calculated as the product of the resistivity and the current density applied to the slide. The current density, expressed in milliamperes per square centimeter, was derived by dividing the current applied per slide by 0.262 cm², the calculated cross-sectional area of the agar gel on the slide.

RESULTS

Typical effects of varying the buffer system are illustrated schematically in Fig. 2, which shows the immunoelectrophoretic separation patterns obtained with the reference antigen in Ionagar using a discontinuous and a continuous buffer system. A considerably longer separation pattern and a better resolution of the individual precipitin bands was obtained with the discontinuous buffer; nine bands could be detected in this system, whereas only seven were seen with the continuous buffer. These differences were recognized consistently with-

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**Fig. 1.** Half-bridge apparatus for determining resistance.

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**Fig. 2.** Influence of buffer systems on separation of mycobacterial reference antigen.
out regard to the type of agar used to prepare the gel.

A comparison of the immunoelectrophoretic patterns obtained when the reference antigen was separated in various agar preparations using the discontinuous buffer system is illustrated in Fig. 3. The agarose preparation produced the longest separation pattern, but its resolution of the anodic components of the reference antigen was less than that observed with the other preparations. The patterns obtained with the Noble and purified agars were of intermediate length and essentially identical. Their resolution of the anodic components was nearly equal to that of the Ionagar preparation, although the bands were somewhat flatter, and they provided the most definite separation of the cathodic components.

The quantitative data from these experiments, as well as from the resistivity determinations, are summarized in Table 1. Regardless of the type of agar employed as the supporting medium, the length of the separation pattern in the discontinuous buffer system was significantly greater ($P < 0.001$) than that in the continuous system. Similarly, considerable differences were noted in the length of the pattern when the reference antigen was separated in the various agar preparations using the discontinuous buffer. The separation length in Ionagar was significantly less ($P < 0.001$) than that of any of the other preparations. The difference between the length of the pattern in agarose and either Noble or purified agar was of questionable significance ($P = 0.025$). No significant difference ($P > 0.10$) was noted between the agar preparations, however, when the continuous buffer system was used. The results of the resistivity determinations, in general, agreed well with separation length measurements. The differences in separation lengths between buffer systems were reflected consistently by the values for resistivity and field intensity, as were the differences noted between the various agar preparations in the discontinuous system. Likewise, the failure to detect significant differences between agar preparations in the continuous system was reflected by the nearly identical values for resistivity and field intensity of these preparations.

The influence of the design of the slide carrier on the electrophoretic separation of the reference antigen is shown in Fig. 4. It was apparent that in the LKB carrier the pattern was longer and the anodic bands were flatter and less well resolved than in the Buchler carrier. These comparisons suggested that slides on the LKB carrier were subjected to a higher current density. This impression was

![Fig. 3. Influence of agar preparations on separation of mycobacterial reference antigen.](image)

**Table 1. Influence of buffer and supporting medium on immunoelectrophoretic separation of mycobacterial reference antigen**

| Conditions of immunoelectrophoretic separation* | Buffer* system | Supporting medium | Resistivity (ohm-cm) | Field intensity (mV/cm) | Separation length+ |
|------------------------------------------------|----------------|-------------------|----------------------|------------------------|-------------------|
| Discontinuous barbital (pH 8.6, 0.027 ionic strength) | 1% Ionagar no. 2 | 536 | 3,409 | 4.57 ± 0.06 |
| 1% Noble agar | 624 | 3,969 | 5.07 ± 0.06 |
| 1% Purified agar | 660 | 4,198 | 5.07 ± 0.06 |
| 1% Agarose | 663 | 4,217 | 5.40 ± 0.17 |
| Continuous barbital (pH 8.6, 0.050 ionic strength) | 1% Ionagar no. 2 | 310 | 1,972 | 3.37 ± 0.06 |
| 1% Noble agar | 309 | 1,965 | 3.47 ± 0.06 |
| 1% Purified agar | 317 | 2,016 | 3.57 ± 0.15 |
| 1% Agarose | 315 | 2,003 | 3.50 ± 0.10 |

* Constant current density of 6.36 mA/cm$^2$ (1.67 mA/slide) applied for 90 min at 25 C.
* Electrolyte vessels contained barbital buffer (pH 8.6, 0.050 ionic strength).
* Values represent mean length of immunoelectrophoretic separation pattern ± standard deviation measured at 24 hr.
confirmed when the electrical analogs of the slide configuration on the carriers were examined, as illustrated in Fig. 5. Thus, although a current of 10 mA was applied to each carrier, it was obvious that each slide on the LKB carrier was subjected to three times as much current as a slide on the Buchler carrier. The current per slide and the current density on the LKB carrier were calculated to be, respectively, 5.0 mA and 19.08 mA per cm², whereas the comparable values on the Buchler carrier were 1.67 mA and 6.36 mA per cm².

**DISCUSSION**

The results of the present study unequivocally demonstrated that variations in separation methodology have both qualitative and quantitative effects on the immuno-electrophoretic separation pattern obtained with the mycobacterial reference materials, as described previously for other systems (1, 2, 7). Although some of these differences may appear to be trivial, the anticipated and potential use of this reference system in mycobacterial immunology, as discussed earlier, seems to require a precise standardization of conditions for electrophoretic separation. For example, to study species-specific antigens or to compare isolated fractions, the position of individual bands within a pattern can serve as a distinguishing feature, and, consequently, even qualitative changes in the separation pattern should be avoided. Differences such as those noted in the position of the cathodic bands between agar preparations (Fig. 3) in this case could complicate the interpretation of results, and differences produced by varying the buffer system (Fig. 2) could completely negate comparisons of antigens. Even though it is unlikely that separation methodology will be varied significantly within a single laboratory, it is necessary that a standardized methodology be adopted or, at least, comparability in methodology be achieved if this reference system is to provide an effective means of communication between laboratories.

A precise definition of separation conditions obviously is needed to develop standardization and comparability in methodology. The results of the present study provided evidence that a valid definition might be obtained by characterizing the separations in terms of field intensity because its derivation is based both on current density and resistivity. On this basis, the significant difference between buffer systems (Table 1) reflected the approximate two-fold difference in field intensity which, in turn, was determined by a similar difference in resistivity and ionic strength of the buffers. Thus, the concentration difference between buffers, rather than the fact that the buffer in the agar and in the electrolyte vessels was different (discontinuous) or the same (continuous), was the significant factor affecting the separation pattern. The differences in patterns obtained in different slide carriers (Fig. 4) can be similarly explained. When the electrical analogs of the chambers were considered (Fig. 5), the field intensities for the Buchler and LKB slide carriers were calculated to be, respectively, 3,408 and 10,277 mV per cm; on this basis, it was quite reasonable to expect that the separation pattern would be longer in the LKB carrier.

The observed differences between agar preparations, however, appeared to be related to differences both in field intensity and purity. Although the differences in separation length (Table 1) agreed reasonably well with the differences in field intensity, the qualitative differences in separation patterns (Fig. 3),
particularly of the cathodic bands, reflected differences in purity and, consequently, in the electroosmotic properties of the agar preparations. In agreement with the result obtained by Wieme (7), agarose, the purest agar preparation, showed the least resolution of the cathodic components; the Noble and purified agars, of lesser purity, resolved them most clearly; and Ionagar exhibited an intermediate degree of resolution.

Obviously, to avoid these potential complications in the use of the reference system, the originally described separation methods and conditions (4) should be used without modification.

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