Improved Antihyaluronidase Test Applicable to the Microtitration Technique

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The incorporation of India ink into the substrate solution in a standard antihyaluronidase test has facilitated the reading of end points in the standard tube dilution test and has made possible an antihyaluronidase microtiter test.

Presently, the laboratory test most frequently utilized in the diagnosis of rheumatic fever is the antistreptolysin O (ASO) test. Elevated ASO titer, however, have been shown to occur in only 80% of patients with acute rheumatic fever. The inclusion of antihyaluronidase (AH) titer along with the ASO titer increased the number of positive sera to 90% (1). The reluctance to run concomitant AH titrations has been due to the difficulty in reading end points (2) as well as to the cost of running two tests. This report describes a minor alteration in a standard AH test which facilitates the reading of end points and permits the adaptation of the standard tube dilution AH test to a more economical microtiter technique.

The antihyaluronidase test measures the antibody titer in a patient's serum to the streptococcal enzyme, hyaluronidase. The hyaluronidase utilized in the test, if neutralized by the patient's serum, will not break down the potassium hyaluronate substrate, which is then able to form a cloudy-white mucin clot upon the addition of acetic acid. The titer of the serum is the reciprocal of the highest dilution which contains a mucin clot. The standardized desiccated components of a commercially available antihyaluronidase test (AHT) kit (Bacto AHT kit; Difco Laboratories, Detroit, Mich.) were utilized. These were reconstituted, with one exception, with distilled water as recommended by the manufacturer. That exception involved reconstitution of the Bacto AHT substrate (potassium hyaluronate) with a dilute India ink solution instead of the distilled water. This solution was made by adding 0.01 ml of India ink (Higgins Ink Co., Brooklyn, N.Y.) to 20 ml of distilled water. Both the tube test and microtiter test were carried out as recommended for the standard tube dilution test by the manufacturer, except the microtiter test utilized microtiter plates, 0.025-ml microdilutors, and 0.025- and 0.050-ml calibrated dropper pipettes (Cooke Engineering Co., Alexandria, Va.), and the volumes involved were one-tenth of those used in the tube dilution test. The test involved incubating a dilution of the patient's serum with streptococcal hyaluronidase at 37 C for 15 min, followed by refrigeration for 10 min. The substrate, potassium hyaluronate, was added and the mixture incubated at 37 C for an additional 20 min and refrigerated for 30 min. Acetic acid (2 N) was added, 0.1 ml in the tube test and 0.025 ml in the microtiter test, and mixed. The tubes or wells were then observed for the presence of a black mucin clot.

The tube dilution and microtiter antihyaluronidase techniques were compared for eight antisera evidencing a wide range of ASO titers (Table 1). Antihyaluronidase titrations in the two techniques were carried out by using substrate reconstituted with either distilled water or the India ink solution. The titers were identical by three of the four methods, with the exception being the microtitration done with substrate reconstituted in distilled water. In this case the small, colorless clot was impossible to see.

Use of the normal substrate solution results in an extremely small, colorless clot which frequently adheres to the side of the tube or well making it difficult to see in the standard tube test and impossible to see in the microtiter technique. The addition of India ink to the potassium hyaluronate solution results in the formation of an easily seen black clot and is an essential addition when the AH test is adapted to the microtiter technique.

The development of a microtiter test for the
Table 1. Comparison of antihyaluronidase titers obtained with the standard and improved antihyaluronidase tests

| Sample | Antihyaluronidase test | Antistreptolysin O | With ink | Without ink | With ink | Without ink |
|--------|------------------------|--------------------|---------|-------------|---------|-------------|
|        | Tube tests             | Microtiter tests   |         |             |         |             |
| 1      | 32                     | 32                 | 32      | 0*          | 50      |             |
| 2      | 128                    | 128                | 128     | 0           | 50      |             |
| 3      | 32                     | 32                 | 32      | 0           | 100     |             |
| 4      | 16                     | 16                 | 16      | 0           | 125     |             |
| 5      | 32                     | 32                 | 32      | 0           | 166     |             |
| 6      | 128                    | 128                | 128     | 0           | 250     |             |
| 7      | 256                    | 256                | 256     | 0           | 500     |             |
| 8      | 2,048                  | 2,048              | 2,048   | 0           | 833     |             |

* It was impossible to see the clot in the microtiter system without the ink and, therefore, the titers would be read as zero.

The determination of antihyaluronidase titers should facilitate a greater use of this technique, resulting in a greater rate of detection of rheumatic fever.

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LITERATURE CITED

1. Stollerman, G. H., A. J. Lewis, I. Schulta, and A. Taranta. 1956. Relationship of immune response to group A streptococci to the course of acute chronic recurrent rheumatic fever. Amer. J. Med. 20:163-169.
2. Wannamaker, L. W., and E. M. Ayoub. 1960. Antibody titers in acute rheumatic fever. Circulation 21:598-614.