Rapid Fluorescent-Antibody Stain Technique with Group A Streptococci

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A rapid fluorescent-antibody stain technique used with young broth cultures of trypsinized group A streptococci is described. Results from 1,214 trypsinized cultures of group A streptococci employing the rapid stain method were equivalent, both in specificity and sensitivity, when compared to results obtained from identical non-trypsinized cultures stained with the longer standard technique of Cherry, Goldman, and Carski. Moreover, the rapid method requires less than 2 min to complete.

Recently, Martin and Bigwood (11) reported that the rapid fluorescent-antibody stain technique of Kellogg and Deacon (7) could be successfully applied to group A streptococci. Subsequently, a pilot study by Freeburg (4) with a rapid fluorescent-antibody stain technique similarly indicated the feasibility of using this method to identify young broth cultures of group A streptococci. However, Freeburg reported only 83% agreement between the rapid method and the longer stain method of Cherry, Goldman, and Carski (1), citing the reduced intensity of fluorescence of organisms obtained with the rapid method as a serious shortcoming.

An attempt to intensify fluorescence of organisms stained with the rapid fluorescent-antibody technique led to the use of trypsin. The effects of trypsin on streptococcal cell walls have been extensively investigated (3, 5, 10, 14). Fox and Stevenson (3) reported that various metabolites, such as glucose, adenosine triphosphate, amino acids, peptides, and divalent ions, appear to transverse the intracellular barrier of streptococci treated with trypsin more rapidly and in greater quantity than in normal streptococci. Although it is true that treatment of group A streptococcal cells with trypsin will destroy the M antigen (10), Wilson, Zimmerman, and Moody (15) were able to type 97% of 322 group A streptococcal isolates after treatment with 5% trypsin for 2 hr by using only the T-typing technique of Moody, Padula, Lizana, and Hall (13), which indicates that the presence of the M antigen is not necessary for typing 97% of group A streptococci cultures. Matsuno and Slade (Bacteriol. Proc., p. 106, 1969) have shown that the location of the antigen in group A streptococci is such that little antibody is formed during immunization. It seemed, therefore, that if one could “expose” the group A streptococcal antigen with trypsin prior to staining, acceleration of the formation of the antigen-antibody complex would be accomplished, intensifying the stain reaction. This study is concerned with such an attempt, with results measured against the standard stain technique of Cherry, Goldman, and Carski (1).

MATERIALS AND METHODS

Antiserum. Flourescein-conjugated anti-group A streptococcus globulin (2) was derived from rabbits immunized with group A streptococci and adsorbed with group C streptococci (Sylvana Corp., Colorado Serum Co.). Fluorescein-conjugated normal rabbit serum was from nonimmunized rabbits, normally containing antibodies against staphylococci and groups of streptococci other than group A (Sylvana Corp.). Nonconjugated normal rabbit serum was also from nonimmunized rabbits (Colorado Serum Co.).

Reagents. The trypsin stock solution consisted of 0.5 g of 1:300 trypsin in 1 liter of Puck’s bicarbonate-free saline solution without antibiotics (prepared by Colorado Serum Co.). The working solution was a 50% stock solution of trypsin in phosphate-buffered saline (PBS). Final pH was 7.2. The PBS consisted of 0.01 M phosphate-buffered 0.85% NaCl (pH 7.2).

Optimum titers of conjugates were determined by staining organisms of groups A, C, and G streptococci with the antisera to be titered. A total of 17 cultures of group C streptococci, 3 cultures of group G streptococci, and 81 cultures of group A streptococci were stained with antisera. By use of the grading method of Moody, Ellis, and Updyke (12), dilutions of antisera which exhibited a 3+ to 4+ reaction with group A streptococci and a 2+ or less stain reaction with other groups of streptococci were considered optimal. Conjugated anti-group A streptococcus globulin dilutions of 1:20 and 1:100 (in PBS) from Sylvana Corp. and Colorado Serum Co., respectively, were used initially. During the latter part of the study, conjugated anti-group A streptococcus globulin mixed in equal parts with normal rabbit serum was employed in the following manner. A 1:50 dilution (in PBS) was mixed in

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equal parts with a 1:20 dilution (in PBS) of normal rabbit serum, resulting in a 1:100/1:40 final dilution. This was found to be optimal for use with the conventional method (1), and a final dilution of 1:60/1:20 was optimal with the rapid method (11). A summary of titer results is given in Tables 1 and 2.

Throat culture specimens submitted by local physicians via the filter-stripe delayed-recovery method of Hollinger and Lindberg (6) were placed on Trypticase Soy agar (BBL) containing 5% sterile defibrinated sheep blood and incubated at 37 C for 18 to 24 hr. Cultures suspected of harboring group A streptococci were then inoculated into 2 ml of prewarmed (37 C) Todd Hewitt broth and incubated for 20 min in a 37 C water bath. The cells were harvested, washed in PBS, and centrifuged for 5 min, and the sediment was smeared on slides especially designed for the fluorescent-antibody technique (Curtin Scientific). Paired slides for each specimen were prepared. The slides were air-dried, fixed in 95% ethyl alcohol for 1 min, rinsed in PBS, and blotted dry. One slide of each pair was stained with the conventional method by placing one drop of either the 1:20 or 1:100/1:40 dilution of conjugated anti-group A streptococcus globulin on one circle and as necessary one drop of a 1:20 dilution of conjugated normal rabbit serum on the other circle as a negative control. [When the anti-group A streptococcus globulin was adsorbed with normal rabbit serum, 1:100/1:40, a negative control was not used.] The slide was allowed to react for 30 min at room temperature in a moist chamber, rinsed in two changes of PBS, rinsed momentarily in distilled water, and then mounted in buffered glycerol and covered with a no. 1 cover slip. Concomitantly, the second slide of each pair was treated with one drop of trypsin (working solution) in PBS, reacted in a moist chamber at 50 C for 1 min, and blotted dry. One drop of either the 1:20 or 1:60/1:20 dilution of anti-group A streptococcus globulin was applied to one circle and as necessary one drop of a 1:20 dilution of conjugated normal rabbit serum was applied on the other circle as a negative control. [When the anti-group A streptococcus globulin was adsorbed with normal rabbit serum (1:60/1:20), a negative control was not used.] The slides were immediately placed in a glass slide carrier in a vertical position, allowing the antisera to drain, leaving only a thin film. After reacting in a moist chamber at 50 C for 30 sec, the slides were rinsed gently in free-flowing warm tap water (taking care to avoid cross-contamination), blotted dry, and mounted as in the conventional method. Each pair of slides was examined with a Reichert fluorescent-antibody Biozet microscope equipped with a 50X special fluoresce oil immersion lens and a monococular 20X ocular lens with a dark-field condenser. The light source was an Osram HBO 200 mercury-arc vapor lamp. A 3-mm BG-12 ultraviolet (UV) pass filter and a OG-1 UV excluding eyepiece filter were used.

Slides were searched for the brightest stained cells or chains of cells. In most cases, the intensity of chains of cells was recorded. In a few cases where only cells were found (when inoculum was from only one or two colonies), the latter stain reaction was recorded.

Those group A streptococcal suspect cultures which yielded negative results with the fluorescent-antibody method were retested with the hot hydrochloric acid method of Lancefield (8, 9). They were found to be cultures of groups B, C, and G streptococci. Also, in the course of the study, positive group A streptococci cultures were spot checked with the Lancefield method.

RESULTS

A total of 2,428 slides or 1,214 cultures were examined; 2,400 slides or 98.98% were in agreement. Intensity of the stain reaction among those in agreement was equal in 2,078 slides or 85.58%.

### Table 1. Determination of optimum titers of group A fluorescent antibody by using conventional stain method

| Streptococcal group tested† | No. of cultures tested | No. of cultures exhibiting fluorescent-antibody reactions |
|-----------------------------|------------------------|--------------------------------------------------------|
|                             | 0 | 1+ | 2+ | 3+ | 4+ |
| A                           | 81 | 0  | 0  | 49 | 32 |
| C                           | 17 | 5  | 6  | 0  | 0  |
| G                           | 3  | 3  | 0  | 0  | 0  |

† For the first three entries (A, C, G), anti-group A streptococcus globulin was adsorbed with group C streptococci (titer 1:20). For the last three entries (A, C, G), anti-group A streptococcus globulin was adsorbed with group C streptococci and diluted with normal rabbit serum (titer, 1:100/1:40).

### Table 2. Determination of optimum titers of group A fluorescent antibody by using rapid stain method

| Streptococcal group tested† | No. of cultures tested | No. of cultures exhibiting fluorescent-antibody reactions |
|-----------------------------|------------------------|--------------------------------------------------------|
|                             | 0 | 1+ | 2+ | 3+ | 4+ |
| A                           | 81 | 0  | 0  | 2  | 45 | 34 |
| C                           | 10 | 6  | 4  | 0  | 0  | 0  |
| G                           | 2  | 0  | 0  | 0  | 0  | 0  |

† For the first three entries (A, C, G), anti-group A streptococcus globulin was adsorbed with group C streptococci (titer, 1:20). For the last three entries (A, C, G), anti-group A streptococcus globulin was adsorbed with group C streptococci and diluted with normal rabbit serum (titer, 1:60/1:20).
Table 3. Results from two fluorescent-antibody stain methods on 1,214 group A streptococcal cultures

| Determination                                      | Rapid method | Conventional method |
|----------------------------------------------------|--------------|---------------------|
| No. of slides processed                            | 1,214 (100.00) | 1,214 (100.00)      |
| No. of slides in agreement                         | 1,200 (98.84) | 1,200 (98.84)       |
| No. of slides not in agreement                     | 14 (1.15)    | 14 (1.15)           |
| No. of slides staining with superior intensity      | 180 (14.82)  | 142 (11.69)         |
| No. of slides staining with inferior intensity      | 0 (0.00)     | 38 (3.13)           |
| No. of positive slides                             | 1,150 (94.72) | 1,150 (94.72)       |

* Numbers in parentheses indicate percentages.

A superior stain intensity was exhibited by 180 slides or 14.82% with the rapid method, whereas with the conventional method 142 slides or 11.69% resulted in a superior stain intensity. A net difference of 38 slides or 3.13% showed superior staining with the rapid method. Both methods yielded an equal number of positive slides (1,150).

An equal number of slides, 14, were not in agreement with their paired slide with either method, resulting in 28 slides or 1.15% not in agreement (Table 3).

Since the possibility remained that the enhanced rapid stain reaction was not due to trypsinization but rather to the less dilute antisera used, 198 slides were prepared from 99 cultures of group A streptococci and stained, both with and without prior treatment with trypsin, by using the rapid method. It was found that 98.98% (98 slides) which had been treated with trypsin exhibited a 3+ or 4+ reaction (0 reaction, 1; 1+, 0; 2+, 0; 3+, 37; 4+, 61), whereas with slides stained without trypsin only 37.37% (37 slides) exhibited a 3+ or 4+ reaction (0 reaction, 3; 1+, 16; 2+, 43; 3+, 30; 4+, 7) when stained with identical antisera (anti-group A streptococcus globulin adsorbed with group C streptococci and diluted with normal rabbit serum, titer 1:60/1:20).

**DISCUSSION**

A rapid fluorescent-antibody stain technique used with young broth cultures of trypsinized group A streptococci is described. In this study it was found that the rapid fluorescent-antibody stain technique could be used to identify young broth cultures of group A streptococci if the streptococcal cells were pretreated with a specially prepared solution of trypsin and digested under the conditions described. Sensitivity of the rapid fluorescent-antibody method is apparently enhanced through the use of trypsin. As measured against the standard stain method of Cherry, Goldman, and Carski (1), 100% specificity was obtained. In addition, the rapidity which characterizes the test results in considerable savings of time, an obvious advantage over the conventional method.

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